Quantitative and spatio-temporal features of protein aggregation in *Escherichia coli* and consequences on protein quality control and cellular ageing

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The aggregation of proteins as a result of intrinsic or environmental stress may be cytoprotective, but is also linked to pathophysiological states and cellular ageing. We analysed the principles of aggregate formation and the cellular strategies to cope with aggregates in *Escherichia coli* using fluorescence microscopy of thermolabile reporters, EM tomography and mathematical modelling. Misfolded proteins deposited at the cell poles lead to selective re-localization of the DnaK/DnaJ/ClpB disaggregating chaperones, but not of GroEL and Lon to these sites. Polar aggregation of cytosolic proteins is mainly driven by nucleoid occlusion and not by an active targeting mechanism. Accordingly, cytosolic aggregation can be efficiently re-targeted to alternative sites such as the inner membrane in the presence of site-specific aggregation seeds. Polar positioning of aggregates allows for asymmetric inheritance of damaged proteins, resulting in higher growth rates of damage-free daughter cells. In contrast, symmetric damage inheritance of randomly distributed aggregates at the inner membrane abrogates this rejuvenation process, indicating that asymmetric deposition of protein aggregates is important for increasing the fitness of bacterial cell populations.

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Introduction

Protein misfolding is an inevitable process in all cells that is enhanced by internal and environmental stress such as heat shock. In response to such problems, cells have evolved powerful protein quality control systems, composed of molecular chaperones and proteolytic machineries, to eliminate misfolded proteins and thereby maintain protein homeostasis. In the cytosol of *Escherichia coli*, the DnaK chaperone with its DnaJ and GrpE co-chaperones and the GroEL–GroES chaperonin have central functions in the refolding of misfolded proteins (Hartl and Hayer-Hartl, 2002). Alternatively, misfolded proteins are degraded by overlapping activities of different ATP-dependent proteases that harbour AAA + chaperone and peptidase components or domains (ClpAP, ClpXP, HslUV, Lon and FtsH).

Despite its adaptability, this proteostasis network represents a delicate equilibrium, which can be perturbed by severe stress, leading to the accumulation of misfolded conformers, which form aggregates. Protein aggregates in *E. coli* were first identified as inclusion bodies resulting from the overproduction of heterologous proteins (Carrio *et al*, 1998) and are even detectable in wild-type cells cultivated at physiological temperatures in the absence of protein overproduction (Lindner *et al*, 2008). Protein aggregation can be reverted in the cytosol of bacteria, yeast and plants by a powerful disaggregation machinery, composed of an Hsp70 chaperone system (DnaK in bacteria) and the AAA + chaperone Hsp104 (ClpB in bacteria) (Glover and Lindquist, 1998). This bi-chaperone system solubilizes and refolds aggregated proteins, an activity that is essential for thermotolerance development (Sanchez and Lindquist, 1990; Weibezahn *et al*, 2004).

However, when the refolding and degradation capacity of the cell is persistently limited, an initial strategy seems to involve the sequestration of aggregated proteins at specific sites, thereby protecting the cellular environment from potentially deleterious protein species (Wigley *et al*, 1999; Kaganovich *et al*, 2008; Kirstein *et al*, 2008). Such sequestration may also allow for an asymmetric inheritance of damaged proteins if the aggregated proteins cannot be eliminated before cell division (Rujano *et al*, 2006; Kaganovich *et al*, 2008). In *E. coli*, protein aggregates are deposited at the polar sites and this spatial sequestration was suggested to allow for asymmetric damage inheritance (Lindner *et al*, 2008; Rokney *et al*, 2009). The underlying mechanisms for the sequestration of aggregated proteins and the resulting consequences for the protein quality control system, however, remain elusive. Furthermore, it is not evident whether asymmetric damage segregation is beneficial compared with symmetric inheritance, as it was so far not possible to manipulate the site of cellular protein aggregation.

Here, we present a rigorous analysis of the principles of protein aggregation caused by physiological heat stress in *E. coli* and the relationship between aggregate deposition and bacterial ageing.

Results

Quantitative analysis of protein aggregation using thermolabile reporters

Heat treatment of *E. coli* cells within their growth temperature range can lead to protein aggregation, providing a means to study the aggregation process at physiological stress conditions. To monitor protein aggregation *in vivo*, we constructed N- or C-terminal fusions between thermostable yellow or cyan fluorescent proteins (YFP, CFP) and thermolabile model proteins (*Photinus pyralis* Luciferase, *E. coli* MetA), which aggregate in *E. coli* at temperatures above 44°C (Gur *et al*, 2002; Weibezahn *et al*, 2004). Immunoblot analysis of cell extracts prior and after heat treatment revealed only single protein bands corresponding to the sizes of the fusion proteins, assuring that fluorescence microscopy images of the fusion proteins reflect the cellular localization of the full-length proteins. To exclude the possibility that protein aggregation is the consequence of protein overproduction, we produced YFP–Luciferase and MetA–YFP from IPTG-controlled expression plasmids only to low/intermediate levels (2800 and 1500 molecules/cell, respectively) that were even below the endogenous levels in case of MetA (Supplementary Figure 1; data not shown). Spectinomycin was added to the cells before heat shock to block *de novo* protein synthesis, ensuring that any differences in cellular localization reflect the re-distribution of pre-existing fusion protein.

YFP–Luciferase and MetA–YFP showed a uniform cytosolic staining at 30°C, whereas after temperature upshift to 45°C, both fusion proteins quantitatively re-localized to form foci at the cell poles (>75% of total YFP fluorescence; Figure 1A and B). For YFP–Luciferase, we determined that foci formation was accompanied by the complete loss of Luciferase activity (data not shown). In contrast, heat-induced polar localization was not observed when YFP alone was produced in control experiments (data not shown), together showing that the observed re-distribution is driven by the thermolabile fusion partner. During a recovery period at 30°C, polar YFP–Luciferase and MetA–YFP foci were reverted to a uniform cytosolic staining and polar foci were no longer observed after 45–60 min. This process was directly coupled to the regain of Luciferase activity, indicating that the disappearance of fluorescent foci reflects ClpB-mediated protein disaggregation. Accordingly, the disintegration of heat-induced YFP–Luciferase foci was not detectable in $\Delta clpB$ mutant cells (data not shown), thereby qualifying YFP–Luciferase and MetA–YFP as valuable reporter to study protein aggregation and disaggregation *in vivo*.

Using a heating device for the microscope, we directly followed the kinetics of YFP–Luciferase and MetA–YFP aggregation in individual cells. The first fluorescent foci of YFP–Luciferase appeared after 2–5 min and aggregation was completed after 10 min; MetA–YFP aggregated slower, exhibiting the first visible aggregates after 4–8 min (Supplementary Figure 2). Statistical evaluation revealed that 62% of all heat-treated cells ($n=200$) exhibited two fluorescent foci at both poles, 22% contained only one

focus at one pole, 1% showed one focus in mid-cell position, whereas $\leq 15\%$ contained three fluorescent foci (Figure 1B). In the latter case, two foci were localized to poles and the third one located at mid-cell, probably representing a future septation site. Aggregation was also analysed without addition of antibiotics and resulted in the same number and localization of fluorescent foci after heat treatment, showing that *de novo* protein synthesis is not required for the polar deposition of aggregates.

EM tomography of protein aggregates

To elucidate the molecular features of the protein aggregates, we used electron tomography (ET) of plastic embedded wild-type and $\Delta clpB$ mutant cells, with the latter expressing YFP–Luciferase, which allows to compare the extent of protein aggregation in cells with and without disaggregating chaperone activity. Aggregates were detected as electron dense deposits close to the poles of heat-treated cells (20 min at 45°C), but not in cells kept at 30°C. Using serial sectioning (Supplementary Movies 1–3), we generated 3D reconstructions of complete cells (Figure 1C). Number and localization of the aggregates detected by EM tomography in the reconstructed cells agreed well with the characteristics of those detected by fluorescence microscopy in the living cells. The 3D reconstructions revealed that protein aggregates have amorphous structures and strongly varying sizes. They also allowed to determine the volumes of the aggregates, which in turn allowed to estimate the number of molecules trapped in each aggregate. The average molecular weight of *E. coli* proteins is approximately 35 kDa (Netzer and Hartl, 1998). A typical globular protein of that size occupies a volume of 4.3×10^{-23} l (Harpaz *et al*, 1994). Under the assumptions that the aggregates contain proteins with similar size average and that the volume of these proteins is not dramatically altered on heat denaturation, we estimate that individual aggregates are composed of approximately 2400–16500 protein molecules. The total number of heat-aggregated proteins within a cell (wild-type or *clpB*-expressing YFP–Luciferase) was less diverse between cells, ranging from 17500 to 33000 aggregated proteins/cell, which corresponds to 1.5–3% of total cytosolic proteins. We note that the presence of YFP–Luciferase did not significantly alter the degree of aggregation of endogenous thermolabile proteins, consistent with its relatively low production level.

To ensure that the preparation of cells for electron microscopy did not affect the existing protein aggregates, we performed in addition cryo-ET of vitreous sections of cells in a near-native state. Using this technique, we obtained similar data with respect to number, size and localization of heat-induced protein aggregates, showing that the reconstruction of *E. coli* cells provides a valuable model for analysing protein aggregation (Figure 1D).

Selective re-distribution of quality control components to polar aggregates

We analysed the localization of the protein quality control machinery prior and after heat treatment using C-terminal fusions of DnaK, DnaJ, ClpB, ClpX, HslU, Lon and ClpP to YFP or CFP. If not stated differently, all fusion proteins were produced from IPTG-controlled expression plasmids in respective knockout cells to approximately wild-type levels (at 30°C) and their functional active state was verified

Figure 1 Heat-induced protein aggregation in *E. coli*. (A) MC4100 cells expressing either YFP-Luciferase or MetA-YFP were grown at 30°C and shifted to 45°C for 20 min. The images show an overlay of YFP signal (false coloured in green) and membrane stain FM4-64 (red). Scale bar: 1 µm. (B) Localization of YFP-Luciferase and MetA-YFP aggregates after heat shock ($n = 200$). Positioning of protein aggregates is indicated with blue dots in the inset. (C) 3D electron tomographic reconstructions of heat-treated, serial-sectioned plastic embedded wild-type and $\Delta clpB$ mutant cells harbouring protein aggregates. $\Delta clpB$ mutant cells were expressing YFP-Luciferase. Cell surfaces are visualized in grey colour and aggregates in red. Scale bar: 100 nm. Quantifications of aggregate size and calculations of the copy numbers of aggregated protein species are given. (D) Obliquely sectioned 7.2 nm thick cryo-electron tomographic slices of vitreous sections of heat-shocked wild-type cells. Various electron dense complexes are visualized in the cytosol (presumably ribosomes), whereas the protein aggregates (indicated through the red dotted line) seem more electron lucent excluding these complexes. Scale bar: 100 nm.

(Supplementary Figure 3). Immunoblot analysis revealed only single protein bands corresponding to the sizes of the fusion proteins. As GroEL cannot be fused to YFP/CFP in its functional active state, we monitored its cellular localization (and that of DnaK for comparison) by immunofluorescence.

We first monitored the localization of YFP/CFP fusions to DnaK, DnaJ and ClpB, which constitute the central bacterial disaggregation machinery (Mogk *et al*, 1999). Although at 30°C the fusion proteins exhibited diffuse cytosolic staining, on heat shock to 45°C, they extensively re-localize to the poles (Figure 2A). Confirming these results, DnaK also exhibited polar localization after heat treatment when analysed by immunofluorescence. GroEL instead did not change its localization on heat stress treatment and remained distributed throughout the cytosol (Figure 2B).

We next analysed the localization of the proteolytic machineries. At 30°C, the fusion proteins to ClpX, HslU and ClpP all exhibited uniform cytosolic staining. The only exception was Lon-YFP, which showed a more condensed

fluorescence coinciding with the DAPI staining of the chromosome that is reminiscent of nucleoid-associated proteins (data not shown). After temperature upshift to 45°C, polar foci were observed for all fusion proteins except Lon-YFP, which did not change its localization (Figure 2C).

Numbers and positions of the fluorescent foci generated by the different chaperone/protease fusion proteins on temperature upshift were comparable with those formed by YFP-Luciferase and MetA-YFP, suggesting that the polar localization of quality control components is directed by the occurrence of protein aggregates (Supplementary Figure 4). Indeed, co-expression of YFP-Luciferase and ClpB-CFP showed complete co-localization of both fusion proteins at polar foci after heat shock (Supplementary Figure 5).

DnaJ, DnaK and ClpB dynamically associate with protein aggregates

The extent of polar localization differed significantly between the individual fusion proteins and was most pronounced in

Figure 2 Re-localization of chaperones and proteases on heat treatment. (A) Indicated chaperone fusion proteins were produced in respective knockout cells, except DnaK-YFP that was produced in wild-type cells to approximately 1000 molecules. Cells were grown at 30°C to mid-logarithmic growth phase and heat shocked to 45°C for 20 min. The images show an overlay of the YFP signal (false coloured in green) and the membrane stain FM4-64 (red). Scale bar: 1 μ m. (B) Wild-type cells were incubated at 30°C or heat shocked to 45°C for 20 min. GroEL and DnaK localizations were determined by immunofluorescence using specific antibodies. Scale bar: 1 μ m. (C) Indicated AAA + protein and peptidase fusions were produced in respective knockout cells that were grown at 30°C to mid-logarithmic growth phase and shifted to 45°C for 20 min. The images show an overlay of YFP signal (false coloured in green) and membrane stain FM4-64 (red). Scale bar: 1 μ m. (D) Calculated ratio of polar and cytosolic fluorescence intensity for the indicated fusion proteins after heat shock. The fluorescence intensities of 20 individual cells harbouring two polar aggregates of the respective fusion proteins were determined.

case of DnaK-YFP and ClpB-YFP ($\geq 75\%$ of total fluorescence) (Figure 2D). These differences in the degree of heat-induced polar fluorescence between refolding (DnaK/ClpB) and degrading machineries (ClpX/ClpP/HslU) may imply differences in their binding kinetics to aggregates. To investigate this possibility, we determined the dynamics of the relevant components at the cell poles using fluorescence recovery after photobleaching (FRAP) experiments (Figure 3). The fluorescence of individual polar foci was bleached followed by a monitoring of the time course of fluorescence recovery. Such experiments were performed using heat-treated cells harbouring fluorescent foci at both poles, thereby allowing for fluorescence recovery through the non-bleached focus and the cytosolic fluorescent fraction. Before heat shock, spectinomycin was added to ensure that the fluorescence recovery is solely resulting from the re-localization of pre-existing fusion protein. The kinetics of fluorescence recovery showed at least two phases. The first phase represented an initial fast recovery because of the re-equilibration of cytosolic fusion proteins within this

compartment. This phase represented a fraction of free diffusing cytosolic proteins (D_f) and was observed to a similar degree (approximately 40% of regained fluorescence) for all analysed fusion proteins. Second was a slower recovery phase that correlated with a loss of fluorescence at the non-bleached focus. This regain in fluorescence includes two steps: the dissociation of the respective fusion protein from the non-bleached focus and the re-association with the bleached focus, thereby representing the mobile aggregate-associated fraction ($M_{\text{aggregate}f}$). We defined the D_f and the $M_{\text{aggregate}f}$ as the mobile fraction (M_f). The immobile fraction (I_f) resulted from non-exchangeable aggregate-associated proteins. We first monitored the recovery of polar fluorescence of YFP-Luciferase aggregates in heat-treated wild-type cells on temperature downshift to 30°C. YFP-Luciferase fluorescence was only regained during the initial fast recovery phase showing that the aggregated molecules are immobile (40% M_f) (Figure 3). A similar result was obtained in case of ClpX-YFP, indicating that the AAA + component of the ClpX/ClpP proteolytic machinery is irreversibly sequestered

Figure 3 Disaggregating chaperones dynamically interact with aggregated proteins. FRAP measurements of Luciferase and chaperone (ClpB, DnaK, DnaJ) YFP fusion proteins were carried out in corresponding knockout strains that were heat shocked to 45°C for 10 min. Analysis of DnaK-YFP was performed in wild-type cells. *De novo* synthesis of fusion proteins was inhibited by addition of 100 µg/ml spectinomycin before heat shock. One out of the two polar fluorescent foci was bleached when cells were shifted back to 30°C for recovery. Representative pre- and post-bleach images of the indicated fusion proteins were recorded at the indicated time periods and recovery curves were calculated based on 10–20 cells.

within the aggregates (Supplementary Figure 6). In case of HslU-YFP, we observed a minor Mf (59% Mf), whereas the majority of HslU-YFP stayed immobile (Supplementary Figure 6). In contrast, a pronounced polar fluorescence was regained for DnaK-YFP, DnaJ-YFP and ClpB-YFP (100% Mf), concomitant with a loss of fluorescence at the unbleached pole, which declined with similar kinetics (Figure 3). This finding shows that the disaggregating chaperones are rapidly recruited from the opposite cell pole and exchange between sites of protein damage within the entire cell. The kinetics of the recovery (Mf) was fast for all three chaperone components, with ClpB being slower than DnaK and DnaJ. Together, these data illustrate that DnaK, DnaJ and ClpB associate dynamically with polar-localized aggregated proteins, whereas the occurrence of ClpX and HslU at the poles rather indicates co-aggregation by binding to misfolded protein species.

Nucleoid occlusion determines polar localization of protein aggregates

As protein aggregates are specifically deposited at cell poles, but are not randomly distributed throughout the cytosol, it seemed an intriguing possibility that a cellular machinery is actively engaged in aggregate deposition, by either transporting aggregates to the poles and/or providing a polar retention system. To initially differentiate between these possibilities, we microscopically followed protein aggregation in real time using YFP-Luciferase and MetA-YFP as fluorescent monitors. In most cells subjected to a temperature upshift to 45°C (76%, $n = 100$), the fluorescent foci formed directly at the poles without showing any movement over longer distance. Still, in a minority of cells (24%), the sites of initial and final positioning of foci differed, and occasionally

(10%) we observed the fusion of smaller foci to bigger aggregates. Thus, a minority of aggregates does not form directly at their final polar destination, but instead appears to move within the cell (Supplementary Movie 4/5).

To detect a possible transport machinery that moves aggregates within the cell, we used several approaches. We first tested whether the cytoskeleton is involved in polar foci formation by inactivating the bacterial actin homologue MreB with the inhibitor A22 (Iwai *et al*, 2002). Treatment of cells with A22 abrogated the helical structure of a YFP-MreB fusion protein without affecting cell shape within the time frame of the experiment. Addition of A22 to the cells before a heat treatment neither changed the number nor the positioning of protein aggregates, largely excluding a function of the cytoskeleton in the aggregation process (Supplementary Figure 7A).

We then investigated whether the polar positioning of aggregates is an active, energy-driven process by treating cells with the uncouplers 2,4-dinitrophenol (DNP) and CCCP or sodium azide, leading to strongly reduced ATP levels (to 20–34% as compared with non-treated cells) before heat shock. However, when monitoring YFP-Luciferase aggregation in such cells, we did not observe differences in the aggregation process (Supplementary Figure 7B–E). In contrast, the solubilization of aggregated proteins by DnaK/ClpB was abolished on ATP depletion by DNP, showing that the disaggregation, but not the aggregation, process requires normal ATP levels.

We also speculated that molecular chaperones are directly involved in aggregate deposition by targeting misfolded protein species to polar sites. Here, we tested for an essential function of the DnaK chaperone machinery in aggregate deposition, as this chaperone system represents the central

holder chaperone in *E. coli* cells at elevated temperatures (Mogk *et al*, 1999). Polar aggregate formation of MetA-YFP was largely unaffected in $\Delta dnaJ$ or $\Delta dnaK$ mutant cells, as dominant polar fluorescent foci were still detectable (Supplementary Figure 8). The existence of additional fluorescent foci in some of these cells is likely caused by the strong increase in protein aggregation and cell size of the filamentous mutant cells (data not shown).

Next, we analysed whether septum formation, which generates cell poles on division, has an impact on the positioning of MetA-YFP aggregates. This was carried out by treating cells with cephalaxin, which inhibits FtsI, thereby causing the formation of filamentous, multi-nucleated cells (Pogliano *et al*, 1997). In cephalaxin-treated cells, multiple MetA-YFP foci were detectable at regular positions within the entire cell body in regions free of bacterial nucleoid (Figure 4A). This finding indicates that the positioning of cytosolic protein aggregates is not restricted to poles and thus not determined by cell division. Instead, it shows that protein aggregates are only formed in the nucleoid-free space.

To directly test whether nucleoid occlusion determines the positioning of aggregates, we sought to monitor protein aggregation in the absence of nucleoids by two strategies. First, we used $\Delta mukB$ cells that frequently produce anucleoid cells because of defects in chromosome segregation (Onogi *et al*, 2000) (Figure 4B). Second, we disrupted the nucleoid architecture by short-term expression (30 min) of the bacteriophage T4 nucleoid disruption protein (Ndd) (Figure 4C). Ndd causes a re-organization of the nucleoid towards the cell periphery, leaving a nucleoid-free cytosol before heat shock (Bouet *et al*, 1996). When MetA-YFP aggregation was monitored in $\Delta mukB$ and Ndd-induced cells, we observed that the absence of nucleoid significantly affected the aggregation process by decreasing the number of aggregates to one per cell and increasing the number of aggregates in mid-cell position (Figure 4B and C). These findings indicate that nucleoid occlusion represents the primary parameter determining both number and final positioning of heat-induced protein aggregates.

We challenged our findings by testing whether a simple physico-chemical model, entirely based on the diffusion and irreversible coagulation of misfolded proteins in combination with DNA-induced occlusion, can sufficiently explain our results. We developed a simulation model that takes into account the diffusion of proteins in the cytoplasm, their irreversible aggregation and the crowding in the bacterium's centre because of the DNA (see Materials and methods for detail). The results of this model are in very good agreement with the experimental data (Figure 4D). We observed almost exclusively a single aggregation focus that dominantly localized to the cell centre when DNA crowding was neglected. In the presence of DNA, the simulations revealed a dominant phenotype with two foci at the bacterial poles. Diffusion and aggregation, guided by the occlusion of DNA in the cell centre, are, therefore, sufficient to explain the experimentally observed phenotypes without the need for additional regulatory elements.

Protein aggregation at the chromosome

Our finding that a rather passive mechanism dictates the deposition site of aggregates raises the question whether alternative sites may also exist, perhaps triggered by aggrega-

tion 'seeds' at specific subcellular structures. We, therefore, targeted thermolabile proteins to either the chromosome or the inner membrane and monitored (i) the aggregation and disaggregation properties of the respective reporter proteins and (ii) the consequences on global protein aggregation.

We first investigated how the association of thermolabile proteins with the chromosome affects their aggregation. CFP-Luciferase was targeted to the DNA by fusing it to the DNA-binding domain of the *lac* repressor (LacI) and after the consequences on protein aggregation. LacI-CFP-Luciferase was specifically directed to the terminus (*ter*) region of the bacterial chromosome located at the mid-cell position (Lau *et al*, 2003), by using a strain harbouring 240 copies of LacI-binding cassettes integrated at the *ter* site. When such cells were grown at 25°C, one fluorescent focus of LacI-CFP-Luciferase was detected at mid-cell position, whereas a uniform cytosolic distribution was observed in control cells lacking LacI-binding sites in the chromosome (Figure 5A; Supplementary Figure 9A). On temperature upshift, no change in LacI-CFP-Luciferase localization was observed, indicating that unfolded LacI-CFP-Luciferase remained associated with the *ter* sites (Figure 5A). In contrast, polar-localized aggregates of LacI-CFP-Luciferase were observed in control cells lacking the LacI-binding sites on the chromosome. This shows that the fused LacI moiety does not alter the aggregation properties of CFP-Luciferase and that the targeting of LacI-CFP-Luciferase to the chromosome restricts its aggregation, if occurring, to this particular site (Supplementary Figure 9A).

Next, we tested the effects of DNA-associated LacI-CFP-Luciferase on the heat-induced aggregation of co-produced cytosolic MetA-YFP or YFP-Luciferase and localization of ClpB-YFP. The localization of the foci of all three cytosolic aggregation reporters was significantly affected by the presence of DNA-bound LacI-CFP-Luciferase. In addition to polar fluorescent foci of the respective fusion proteins, we observed additional fluorescent spots at mid-cell, which showed complete co-localization with LacI-CFP-Luciferase (Figure 5B, C and D; Supplementary Figure 9B). Together, these results illustrate that protein aggregation can also occur at sites other than cell poles, with substantial effects on the aggregation characteristics of other cytosolic proteins.

Protein aggregation at the inner membrane

In an alternative approach, we attached YFP-Luciferase to the inner membrane by fusing it N-terminally with the first and second transmembrane helices of MalF, yielding 2TM-YFP-Luciferase. The 2TM-YFP-Luciferase was produced to similar levels as compared with cytosolic YFP-Luciferase and exhibited a homogeneous membrane staining before heat shock (Figure 6A; Supplementary Figure 1). On temperature upshift to 45°C, multiple fluorescent foci were formed at the membrane along the entire length of the cell without apparent enrichment at polar regions. In contrast, a 2TM-YFP fusion protein still exhibited a homogenous membrane staining after heat shock (Supplementary Figure 10A). The majority of cells expressing 2TM-YFP-Luciferase (80%, $n = 200$) exhibited more than three aggregates after heat shock, revealing significant differences between the aggregation of cytosolic and membrane-anchored YFP-Luciferase (Figure 6A). The aggregates were randomly distributed throughout the membrane as revealed by recording z-stacks

with 200 nm distances (Supplementary Figure 10B). The membrane-associated protein aggregates remained substrates for the cytosolic protein quality control machinery as aggregated 2TM-YFP-Luciferase was still reactivated in a ClpB-dependent manner, restoring a uniform membrane staining at the end of a recovery phase at 30°C in wild-type, but not $\Delta clpB$ mutant cells (Figure 6A; Supplementary Figure 10C). Thus, dramatic changes in the cellular sites of protein aggrega-

tion do not affect ClpB-mediated protein disaggregation. These data furthermore indicate that ClpB can solubilize membrane-tethered aggregates without extracting them from the bilayer, most likely by using the partial substrate-threading mechanism described earlier (Haslberger *et al*, 2008).

The fact that thermally denatured proteins can in principle form distinct aggregates at both cell poles and the inner membrane raises the question as to the relationship between

Figure 5 DNA-anchored LacI-CFP-Luciferase aggregates capture misfolded cytosolic proteins. **(A)** LacI-CFP-Luciferase was produced in AB1157 cells harbouring multiple copies of lac operators at the terminus region (*ter*) of the bacterial chromosome. The localization of LacI-CFP-Luciferase was monitored at 25°C and after heat stress (45°C). The images show an overlay of the CFP signal (blue) and the membrane stain FM4-64 (red). Numbers and positionings of LacI-CFP-Luciferase foci were determined at both temperatures ($n = 100$, right panels). Scale bar: 1 μ m. **(B)** MetA-YFP and LacI-CFP-Luciferase were co-produced in AB1157 cells and images of the respective fusion protein were recorded at 30°C and after heat shock (45°C, 20 min). The overlay (merge) of both images reveals co-aggregation of both model proteins at the *ter* region on heat stress. Membranes were stained with FM4-64. Scale bar: 1 μ m. **(C)** ClpB-YFP and LacI-CFP-Luciferase were co-produced in AB1157 cells. Images of the respective fusion proteins were recorded prior (25°C) and after heat shock (45°C, 20 min), revealing re-localization of ClpB-YFP to the *ter* region of the chromosome (see merge). **(D)** The localizations of stress-induced MetA-YFP or ClpB-YFP foci, generated on co-production of LacI-CFP-Luciferase in AB1157 cells, were quantified ($n = 100$). The positioning of protein aggregates is indicated (white dot: co-localization; yellow dot: exclusive MetA-YFP or ClpB-YFP localization).

Figure 4 Nucleoid occlusion determines the positioning of protein aggregates. **(A)** Bacterial nucleoids and heat-induced MetA-YFP aggregates do not co-localize in filamentous cells. Filamentation of cells producing MetA-YFP was induced by addition of cephalixin. Cells were either kept at 30°C or shifted to 45°C for 20 min. Images were recorded and show membrane staining using FM4-64 (red), DNA staining using DAPI (blue) and the MetA-YFP signal. A merge of the individual images is given. Scale bar: 1 μ m. **(B)** Nucleoid-free $\Delta mukB$ cells predominantly contain only one stress-induced MetA-YFP protein aggregate at either mid-cell or polar position. $\Delta mukB$ cells and isogenic YK1100 control cells producing MetA-YFP were grown at 37°C and shifted to 45°C for 20 min. Anucleoid cells were spontaneously formed in the DNA segregation-deficient $\Delta mukB$ strain. Images were recorded as described in **(A)**. Scale bar: 1 μ m. Numbers of fluorescent MetA-YFP foci per cell and their respective localizations were determined ($n = 100$, lower panel). The positioning of protein aggregates is indicated (blue dots). **(C)** Re-localization of the bacterial nucleoid to the inner membrane by the nucleoid disruption protein (Ndd) leads to the formation of one stress-induced MetA-YFP aggregate at the mid-cell or the polar region of cells. BL21 Rosetta cells producing MetA-YFP and harbouring a plasmid encoding for Ndd were grown at 30°C to mid-logarithmic growth phase. The bacterial culture was splitted and Ndd production was induced in one population by addition of 1 mM IPTG for 30 min before heat shock (+ Ndd). Images were recorded as described in **(A)**. Scale bar: 1 μ m. Numbers of fluorescent MetA-YFP foci per cell and their respective localizations were determined ($n = 100$, lower panel). The positioning of protein aggregates is indicated (blue dots). **(D)** The results of the biophysical model (coloured lines) on the number of protein aggregates and their cellular localization are in favourable agreement with the experimental data (grey bars). The average steady state after 10^4 proteins were allowed to cluster in the cytoplasm over a time course of a few minutes in the presence (red) and absence (blue) of an occluding nucleoid ($n = 50$ independent simulation runs) yield a very good overlap with the experimentally observed phenotypes (shown below the plots).

Figure 6 Membrane-anchored 2TM-YFP-Luciferase forms multiple aggregates on heat shock and reprogrammes aggregation of cytosolic proteins. **(A)** 2TM-YFP-Luciferase was produced *in* wild-type and $\Delta clpB$ mutant cells. Images were recorded before heat shock (0 min), after temperature upshift to 45°C (10 min) and at the end of a recovery period at 30°C (90 min). Before heat shock, protein synthesis was stopped by addition of rifampicin (50 μ g/ml). The number of membrane-localized 2TM-YFP-Luciferase foci generated on heat stress was quantified ($n = 200$, right panel). Scale bar: 1 μ m. **(B)** Luciferase-YFP and 2TM-CFP-Luciferase were co-produced in wild-type cells and images of the respective fusion protein were recorded at 30°C and after heat shock (45°C, 20 min). The overlay (merge) of both images reveals co-aggregation of both model proteins at high temperatures. Scale bar: 1 μ m. **(C)** ClpB-CFP and 2TM-YFP-Luciferase were co-produced in $\Delta clpB$ cells. Images of the respective fusion proteins were recorded prior (30°C) and after heat shock (45°C, 20 min), revealing co-localization of fluorescent foci after heat stress at the membrane (see merge). Scale bar: 1 μ m.

these two aggregate forms. To address this issue, we co-produced membrane-anchored 2TM-CFP-Luciferase and cytosolic YFP-Luciferase in the same cells and analysed the localization of the respective aggregates after heat treatment (Figure 6B). Although the number and positioning of membrane-anchored 2TM-CFP-Luciferase remained unchanged, a complete re-targeting of cytosolic YFP-Luciferase aggregation was observed. YFP-Luciferase formed multiple fluorescent foci at the inner membrane, which entirely co-localized with 2TM-CFP-Luciferase. The 2TM-CFP-Luciferase aggregates, therefore, act as aggregation ‘seeds’, which attract cytosolic misfolded YFP-Luciferase, thereby preventing aggregate formation at the cell poles. Such re-targeting was also observed when 2TM-CFP-Luciferase was co-produced with cytosolic MetA-YFP (Supplementary Figure 10E), again showing the high co-aggregation potential of structurally unrelated proteins. The generality of this observation was further substantiated by the finding that the aggregation of denatured 2TM-YFP-Luciferase results in the re-targeting of ClpB-CFP, which serves as a marker for the positioning of all aggregated *E. coli* proteins, to the inner membrane and a complete co-localization with fluorescent foci of 2TM-YFP-Luciferase (Figure 6C).

To explain this phenomenon, we considered the possibility that the intrinsic aggregation kinetics of the chosen model protein (Luciferase), which is faster than that of MetA (see above) and perhaps of other cellular proteins, is responsible for this ‘seeding’ effect. We thus performed the same type of experiments by attaching MetA instead of Luciferase to the membrane. However, the co-production of 2TM-CFP-MetA also completely changed the localization pattern of cytosolic YFP-Luciferase and ClpB-YFP after heat stress (Supplementary Figure 10F and G). It thus seems that the existence of membrane-attached aggregation seeds is responsible for

the global re-targeting of aggregates of cytosolic proteins and the quality control machinery.

Asymmetric but not symmetric damage inheritance allows for rejuvenation of daughter cells

We finally investigated the relationship between the polar aggregation of cytosolic proteins and the process of cell division. We frequently observed that when two fluorescent polar foci are present in a single cell, they exhibit differences in brightness, implying different degrees of protein aggregation at the respective sites (Figure 7A). We wondered whether an asymmetry of cell poles (old versus young pole) is the basis for this observation, as it has been recently shown that *E. coli* small heat shock proteins, used as an indirect marker for protein aggregation, preferentially associate with the old-cell pole in non-heat-shocked cells at 37°C (Lindner *et al*, 2008). We, therefore, monitored protein aggregation immediately after cell division, enabling us to discriminate between old- and new-cell poles. In these experiments, we applied a temperature upshift to 42°C instead of 45°C. Such milder stress conditions increased the number of cells containing only a single polar aggregate, which facilitates to differentiate between protein aggregation at old- or new-poles. An intriguingly high number of cells (88%, $n = 100$) contained fluorescent YFP-Luciferase or ClpB-YFP foci exclusively at old poles, whereas the majority of the new poles remained free of visible protein aggregates (Figure 7A).

This result raises the issue as to the relationship between polar aggregate formation and bacterial ageing, which is defined as a decline in bacterial growth rates with time (Stewart *et al*, 2005). In starving *E. coli* cells, increased levels of aggregated proteins correlate with increased cellular senescence (Maisonneuve *et al*, 2008). Furthermore, the existence of polar-localized aggregates in cells correlates

Figure 7 Asymmetric distribution of protein aggregates allows for the rejuvenation of damage-free new-pole daughter cells. **(A)** Stress-induced YFP-Luciferase and ClpB-YFP foci preferentially localize to the old-cell poles of wild-type cells. Division of cells was monitored before heat stress (42°C, 10 min), allowing for the identification of old- and new-cell poles. Old-cell poles are labelled with an arrow. The localization of stress-induced YFP-Luciferase or ClpB-YFP foci at old and new poles were quantified ($n = 100$, right panel). Scale bar: 1 μm . **(B)** The presence of polar-localized or membrane-anchored protein aggregates diminish the growth rate of *E. coli* cells. Comparison of the normalized growth rates for new- and old-pole cells expressing either YFP-Luciferase or 2TM-YFP-Luciferase. Growth rates of 30 colonies were determined for old- and new-pole cells that were continuously cultivated at 30°C for four generations. Alternatively, the formation of protein aggregates was induced by heat shock (45°C, 20 min) and growth rates were recorded on return to 30°C, accordingly. The average growth rate within each generation was normalized to 1. Old-pole cells harbouring polar YFP-Luciferase aggregates and derived aggregate-free new-pole cells differ significantly in their growth rates ($***P < 0.01$). In addition, new-pole cells expressing 2TM-YFP-Luciferase or YFP-Luciferase show significant differences in their growth rates ($***P < 0.01$). **(C)** Determined absolute growth rates of old- and new-pole populations expressing the indicated fusion proteins (non-heat shocked: 30°C, transiently heat shocked: 45°C). The mean growth rate of the respective entire bacterial population was calculated. Standard deviations are given.

with a reduced growth rate at 37°C, reflecting bacterial ageing (Lindner *et al*, 2008). It is unclear whether the same correlation also holds true for heat-induced protein aggregates. To determine the consequences of an asymmetric deposition of aggregated proteins after heat treatment on ageing, we produced YFP-Luciferase in $\Delta clpB$ mutant cells, enabling us to analyse by time-lapse microscopy the influence of protein aggregates on bacterial growth during the recovery phase for various generations (Supplementary Figure 11). As individual colonies showed variations in their generation times, we normalized the average growth rate, for better comparison, within each generation to 1. When continuously grown at 30°C, the lineage of $\Delta clpB$ cells, which inherited the old pole

exhibited a continuous decline of growth rates when followed for four generations (Figure 7B). In contrast, cells that inherited new poles (generated by septum formation during cell division) regained higher growth rates (0.0246/min compared with 0.0225/min, corresponding to generation times of 41 and 44 min) on cell division compared with the old pole mother cell, thus exhibiting 'rejuvenation' in agreement with earlier findings (Stewart *et al*, 2005). In the additional presence of protein aggregates at the old poles of heat-treated mother cells, the difference in growth rates between old- and new-pole cells became much more apparent (0.0211/min compared with 0.0255/min) (Figure 7B and C). This finding suggests, in agreement with earlier data, that the deposition

of heat-induced protein aggregates at the cell poles accelerates bacterial ageing, but also allows for rejuvenation of damage-free new-pole cells on cell division.

To provide direct evidence for a physiological relevance of an asymmetric inheritance of protein aggregates, we tested whether the bacterial ageing phenotype is solely depending on the polar deposition of aggregates. To this end, we expressed 2TM-YFP-Luciferase in $\Delta clpB$ mutant cells, causing re-targeting of heat-induced protein aggregates from polar sites to random positions at the inner membrane (Figure 6A). This experimental setup enabled us to follow the consequences of a symmetric inheritance of protein aggregates on bacterial ageing. The altered localization of aggregates had a dramatic impact on bacterial growth rates, as the differences in growth rates between old- and new-pole cells were no longer increased (Figure 7B). The biggest differences in growth rates were noticed for new-pole cells that were derived from mother cells harbouring either polar or randomly distributed aggregates. New-pole cells that lost polar aggregates through asymmetric inheritance grew significantly faster than new-pole cells that still retained damaged proteins because of the symmetric inheritance of randomly distributed, membrane-associated protein aggregates (0.0255/min compared with 0.0225/min) (Figure 7C). This finding for the first time provides direct evidence that the deposition of aggregated proteins at polar sites has unique beneficial consequences for the ageing of a bacterial cell population.

Finally, we questioned whether the asymmetric deposition of aggregated proteins at old-pole cells, which is key for asymmetric damage inheritance, is also based on nucleoid occlusion or relies on additional mechanisms that confer specificity towards the old pole. A nucleoid occlusion scenario suggests that the noticed asymmetry relies on a slightly asymmetric distribution of the bacterial chromosome within the cell, leaving more space for aggregate deposition at the old pole. In consequence, an asymmetric localization of protein aggregates at old poles should no longer be observable in nucleoid cells or in cells harbouring a nucleoid in a more condensed state. We first monitored the polar deposition of MetA-mCherry aggregates in anucleoid $\Delta mukB$ cells and analysed those cells that still contained polar aggregates. In such experiments, old-pole cells were identified by co-producing Tsr-YFP, which acts as a specific marker for the old pole (Ping *et al*, 2008). In the absence of a bacterial chromosome, an equal distribution of protein aggregates at old- (53%) and new-pole cells (47%) was observed (Supplementary Figure 12A and C; $n=200$). We then monitored protein aggregation in the presence of chloramphenicol, which leads to condensation of the bacterial nucleoid, leaving more space at both poles (Sun and Margolin, 2004). Such conditions again abrogated the preferential deposition of aggregated proteins at the old pole (54%), compared with the new pole (46%) (Supplementary Figure 12B and C; $n=100$), thereby providing further evidence that nucleoid occlusion not only determines the polar localization of protein aggregates, but also their asymmetric deposition at old poles.

Discussion

Our study provides a detailed analysis of (i) the quantitative and spatio-temporal features of protein aggregation in *E. coli*

cells subjected to heat shock, (ii) the protein quality control machinery that responds to this challenge, (iii) the mechanisms driving aggregate formation at specific cellular sites and (iv) the physiological relevance of aggregate deposition for bacterial ageing. Together, this provides a framework for the mechanistic understanding of the cellular events leading to and strategies coping with protein aggregation.

Although the heat treatment at 45°C used for our experiments is within the growth temperature range of *E. coli*, it is severe enough to generate microscopically visible protein aggregates in wild-type cells, which typically form foci at the cell poles, in agreement with earlier findings (Lindner *et al*, 2008; Rokney *et al*, 2009). Further analysis by cryo-ET revealed that these aggregates exhibit large heterogeneity in size, whereas the total volume of all aggregates per cell, and thus the total number of aggregated proteins, is more similar between cells. On the basis of the determined aggregate volumes, we estimate that 1.5–3% of total cytosolic *E. coli* proteins, corresponding to approximately 17500–33000 molecules, aggregate on heat stress in wild-type cells. Although this is a rather small fraction of total cytosolic protein, it suffices to elicit a strong heat shock response, showing the capacity of this stress regulon to detect even small alterations in proteostasis. The aggregates appear amorphous, suggesting that their formation is driven by non-specific, presumably hydrophobic interactions between misfolded stretches of proteins that may be structurally unrelated. Such formation of mixed aggregates is supported by the observed, surprisingly extensive co-aggregation of different thermolabile aggregation reporters, even if they have different cellular localizations (Figures 5 and 6).

How are misfolded cytosolic proteins targeted to the polar dumping sites in which aggregates are deposited? We did not obtain any evidence for an active, energy demanding transport process, as ATP depletion did not affect protein aggregation. Furthermore, aggregate positioning was neither dependent on MreB, a central component of the bacterial cytoskeleton, nor on DnaJ, DnaK and ClpB, largely excluding a function of the cytoskeleton and the protein quality control machinery in targeting misfolded proteins to the poles. The easiness by which cytosolic protein aggregation is re-targeted by membrane or chromosome-attached misfolded proteins provides a further argument against an active guidance of protein aggregates to poles. Together, these findings strongly argue against an active transport of misfolded or aggregated proteins to the poles. This is in conflict to a recent study, claiming that the polar deposition of aggregated proteins in *E. coli* is mediated by an energy-dependent process involving DnaK and MreB (Rokney *et al*, 2009). We cannot offer an easy explanation for these discrepancies, as the experimental setups used in both studies are rather similar and rely on thermolabile fluorescent reporters. We note that the Rokney study uses an unusual buffer (10 mM Tris-HCl, pH 8.0; 10 mM MgSO₄) for resuspending the cells before performing microscopy, and does not include time-lapse microscopy, which is mandatory for following the destiny of individual aggregates.

We instead provide evidences that nucleoid occlusion is the main driving force, which determines number and positioning of protein aggregates in *E. coli*. First, both parameters were significantly altered in cells lacking a nucleoid or harbouring the chromosome in a different structural state.

Second, filamentous cells develop multiple aggregates at nucleoid-free non-polar sites within the entire cell body. Nucleoid occlusion is thus not only necessary, but also sufficient to determine the positioning and number of protein aggregates in the cytosol. This function of the chromosome is further supported by a mathematical model that exclusively relies on a simple geometric occlusion mechanism. On this basis, the model correctly predicts the formation of protein aggregates at polar sites, in perfect quantitative and qualitative agreement with our experimental findings.

The model also predicts that protein aggregation at the membrane always outcompetes bulk protein aggregation in the cytosol. Even under crowded conditions, the bulk diffusion of unfolded proteins is fairly rapid leading to frequent collisions of unfolded proteins with the cellular membrane. If membrane-resident aggregation seeds reside in the membrane patch at which such a collision occurs, the soluble unfolded proteins are irreversibly recruited to the membrane. Even if a larger cluster of unfolded proteins forms in the cytosol, it will fairly rapidly (i.e. within less than a minute) get recruited to the membrane because of its diffusive motion. Hence, rapidly more and more material is irreversibly sequestered to the membrane, thereby forming the dominant fraction of large aggregates in agreement with our findings (Figure 6B and C). We note that in wild-type cells that do not produce a membrane-anchored aggregation-prone protein, the thermolabile proteins of the cytosol are not targeted to the inner membrane, suggesting that only subcritical amounts of inner membrane proteins are prone to aggregation under such conditions.

The mathematical model can even account for the noticed preferential deposition of misfolded proteins at the old pole when an asymmetric positioning of the nucleoid, perhaps resulting from the division process, is assumed. A more spacious cytosolic region at one pole will increase the number of accumulating misfolded proteins, which may influence the onset of aggregate formation. Indeed, an asymmetric deposition of misfolded proteins was no longer observed when the nucleoid was forced into a more condensed state, for example by addition of chloramphenicol, leaving more space at both poles.

E. coli thus uses a rather passive mechanism to deposit aggregated proteins, in contrast to eukaryotic cells. The localization of aggregates in *S. cerevisiae* (JUNQ/IPOD) or mammalian cells (aggresomes) depends on the cytoskeleton network (Garcia-Mata *et al*, 1999; Kaganovich *et al*, 2008) and involves an active transport of aggregates to their final destination (Kawaguchi *et al*, 2003). Thus, pro- and eukaryotes developed fundamentally different strategies to control protein aggregation. These differences likely arose from the less complex cellular architecture and the much smaller size of prokaryotes, which thereby may not necessitate an active process to ensure the deposition of damaged proteins at specific sites.

What is the physiological relevance of the polar deposition of protein aggregates? We show that the presence of heat-induced, polar aggregates has a negative impact on bacterial growth rates. Similar findings have been recently reported by Taddei and co-workers, showing that non-stressed *E. coli* cells harbouring an aggregation reporter exhibit a reduced growth rate (Lindner *et al*, 2008). The polar localization of aggregates might help cells to regain

faster growth rates, as it allows for the generation of damage-free poles and cells by cell division. In the case of cells harbouring a single aggregate at the old pole, it allows for the generation of a heterogeneous cell population by division, creating new-pole cells that can recover faster from stress situations at the expense of old-pole cells, which retain the main damage. Importantly, this prediction is confirmed by our finding that symmetric damage distribution largely prevents rejuvenation of new-pole cells (Figure 7B and C). This finding provides for the first time direct experimental evidence for the theoretical prediction that asymmetric distribution of damage is beneficial for a cell population by allowing for faster recovery of new-pole cells from stress, which could represent an important growth advantage in a competitive microbial environment (Ackermann *et al*, 2007). Conversely, there may be an evolutionary driving force to avoid non-polar sites of protein aggregation, for example at the inner membrane. It is tempting to speculate that membrane proteins with domains exposed to the cytosol evolved to be more resistant towards thermal unfolding compared with cytosolic proteins, thereby preventing the generation of aggregation seeds. The 2TM-Luciferase-YFP and 2TM-MetA-YFP model constructs, which we used in this study, show that aggregation of membrane-associated proteins is, in principle, possible. The solubilization of these membrane-localized aggregates strictly depended on ClpB, which gets targeted to membrane-associated foci. Our observation that stress-induced ClpB-YFP foci are not formed at the membrane of wild-type cells shows that endogenous membrane proteins do not aggregate on heat stress.

Many components of the protein quality control machinery react to heat stress by re-localization to polar foci. A rapid re-localization of the major cellular fraction of DnaK, DnaJ and ClpB confirms their central function in protein disaggregation. FRAP analysis revealed that these proteins remain highly dynamic and thus are reversible associated with aggregates. A more complicated picture emerged for the proteolytic systems, which in part also re-localized to the polar foci, as not all components seem to represent functional interactions with aggregates. According to FRAP analysis, ClpX and HslU become immobilized at foci and, therefore, rather co-aggregate with misfolded proteins, consistent with their inability to degrade aggregated proteins *in vitro* (jointly with ClpP and HslV, respectively; data not shown). Thus, co-localization or co-sedimentation of chaperones and proteases with protein aggregates is an insufficient criterion for defining a functional role in aggregate handling.

Interestingly, GroEL and Lon, representing important functions in the folding and degradation of misfolded proteins (Chung and Goldberg, 1981; Kerner *et al*, 2005; Chapman *et al*, 2006), do not re-localize on heat stress and remain distributed throughout the cytosol. For GroEL, this is explained by its mode of action, which relies on the encapsulation of single non-native proteins, and hence represents a 'downstream' activity after the DnaK/DnaJ/ClpB-mediated solubilization of aggregated proteins. Solubilized proteins likely diffuse rapidly throughout the entire cytosol and thus do not require an enrichment of GroEL at polar regions after heat stress. GroEL thereby remains available for its house-keeping function in the folding of newly synthesized proteins. Similarly, the fast diffusion of misfolded species does not necessitate a polar localization of Lon, which can only act

on soluble non-native proteins, but not on heat-aggregated proteins *in vitro* (data not shown).

Summarized, we provide for the first time a rigorous, quantitative analysis of protein aggregation in a single cell. It will be an interesting future topic to compare the features of the aggregation/disaggregation processes in *E. coli* with those of the related processes in other organisms, which have different sets of cytosolic chaperones including the lack of a powerful disaggregation activity.

Materials and methods

Plasmids and strains

All *E. coli* K12 strains and plasmids used in this study are listed in Supplementary Tables 1 and 2. Fluorescent fusion proteins were constructed by fusion PCR. Target genes and *mcherry* or *yfp/cfp* were amplified using primers with complementary overlaps, encoding a 5x glycine linker. The fusion genes were inserted into the listed expression plasmids (Supplementary Table 1).

Growth conditions

Cell cultures were grown, if not indicated otherwise, at 30°C in LB media to an OD₆₀₀ of 0.6–0.8. For heat treatment, cells were shifted to 45°C for 10–20 min. When appropriate, antibiotics were added to standard concentrations. Gene expression was induced on addition of the indicated concentrations of arabinose or IPTG (Supplementary Table S2). All fluorescent chaperones/peptidase fusion proteins were produced at wild-type level. To stop protein translation, spectinomycin (100 µg/ml), rifampicine (50 µg/ml) or tetracycline (25 µg/ml) was added 10 min before starting the respective experiment.

ATP depletion experiments were performed in MOPS-based minimal media with succinate as carbon source (Neidhardt *et al*, 1974). A total of 10 mM DNP was added to the growth medium 30 min before heat shock. For energy depletion experiments with CCCP (20 µM) or natriumazide (0.5–1% (w/v)), cells were resuspended in PBS, incubated for 5 min with uncoupler and washed with PBS before applying a heat shock. Intracellular ATP levels were determined using a luciferin/luciferase-based assay as described (Yang *et al*, 2002).

E. coli Δ *mukB* cells were grown in MCAT media (Onogi *et al*, 2000) and A22 was added 20 min before heat shock.

Enzymatic assays and determination of protein copy numbers

Luciferase activities were determined from 200 µl cell suspensions in a luminometer (Lumat berthold LB9501) as described by Schröder *et al* (1993). The cellular levels of YFP fusion proteins were determined by measuring the YFP fluorescence intensity from total cell extracts in a luminescence spectrometer (Perkin Elmer). A standard curve was generated from cell lysates spiked with different quantities of purified YFP (details are included in the supplements).

Membrane and DNA staining

For membrane staining, the dye FM4-64 (2 µg/ml, Molecular probes) was added to the culture 20 min before start of the experiment. For DNA staining, cells were fixed for 5 min on ice with 2.8% (v/v) formaldehyde and 0.04% (v/v) glutaraldehyde. Next, fixed cells were incubated with 0.3 µg/ml DAPI for 10 min. Three washing steps with 1 ml tethering buffer (TEB) were carried out to remove the fixatives and free DAPI.

Microscopy and cryo-ET

For snapshot imaging, cells pellets were resuspended in TEB (Block *et al*, 1983) and immobilized on agarose pads. Agarose pads were sealed with Apiezon grease and covered with cover slips. For long time-lapse imaging with growing cells, the cells were spotted onto agarose pads that contained 15% (v/v) LB media and were sealed in a custom-made aluminium slide using cover slips on each side. For short time-lapse experiments, cells were collected, washed in TEB and placed on agarose pads without media. The slide was either placed into a custom-made temperature-controlled holder that was connected to a water bath (Lauda Ecoline Star edition, for FRAP experiments), a metal holder that was connected to a peltier element (Oven industries, for studying protein aggregation) or a Toka Hit temperature control chamber (to study the inheritance of

protein aggregates). For fluorescence microscopy, various microscopes and settings were used as specified in Supplementary data.

Methods for cryo-ET and for the generation of cell sections are described in Supplementary data.

Immunofluorescence

Cells were fixed and stained as described (Teleman *et al*, 1998; Sourjik and Berg, 2000). Fixed cells were directly immobilized on poly-L-lysine-coated slides. Incubation with affinity-purified antibodies (α -GroEL (1:200 dilution) or α -DnaK (1:1000 dilution)) and secondary antibody (1:500 dilution Alexa 546 anti rabbit, Molecular Probes) was carried out for 1 h at room temperature in 2% (w/v) BSA containing PBS buffer with (GroEL) or without (DnaK) 0.05% (v/v) Tween. After washing with PBS, cells were embedded in mounting solution (1 mg/ml p-phenylenediamine, 90% glycerol and 5% PBS) before imaging. Incubation of MC4100 cells with the secondary antibody alone did not result in any fluorescence signal. In addition, GroEL and DnaK antibodies did not show background staining in GroEL-depleted MGM100 or Δ *dnaK52*.

FRAP experiments

For FRAP experiments, cells were grown at 30°C in TB media to an OD₆₀₀ of 0.5. Cells were immobilized on poly-L-lysine-coated slides and incubated with TEB containing 100 µg/ml spectinomycin and 10% (v/v) TB media. Cells were heat shocked for 10 min at 45°C and shifted back to 30°C. After 10 min, FRAP measurements were started. For each chaperone-YFP protein fusion, 10–20 individual cells were measured. Fluorescence of polar protein aggregates were bleached with two 0.336 s scans at 50% laser intensity, pre- and post-bleach images were acquired with 3–5% laser intensity using bidirectional scanning. Post-bleach images consisted of 30 images, 10 images with 0.336 s, 10 images with a time interval of 3 s, followed by 10 images with a time interval of 30 s. Measurements were performed on a laser scanning confocal microscope (Leica TCS SP2) equipped with an FRAP software module.

Image processing, data analysis and spatio-temporal model for protein aggregate formation

Image processing was carried out using Image J (W Rasband, <http://rsb.info.nih.gov/ij/>) software. Statistics and plotting of the data were performed with Excel. Quantification of polar and cytosolic fluorescence intensities was calculated from 20 cells using the following formula: % polar fluorescence = fluorescence of the region of interest (ROI) – background ROI/fluorescence of the whole cell background $\times$ 100. For FRAP experiments, the fluorescence intensity of the whole cell and the polar ROI was determined over time. Relative fluorescence intensity of ROI was normalized to the relative ROI before bleaching. *Mf* and *If* were calculated from the corresponding recovery curves. For time-lapse experiments that involved cell growth, old- and new-pole cells were tracked manually and growth rates were measured from 30 colonies/strain by determining the time between two cell divisions. The development of the spatio-temporal model for protein aggregate formation is described in detail in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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