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***Title*:**

Direct assessment in bacteria of prionoid propagation and phenotype selection by Hsp70 chaperone

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***Running title*:** Dynamics of a bacterial prionoid *in vivo*

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Summary

Protein amyloid aggregates epigenetically determine either advantageous or proteinopathic phenotypes. Prions are infectious amyloidogenic proteins, whereas prionoids lack infectivity but spread from mother to daughter cells. While prion amyloidosis has been studied in yeast and mammalian cells models, the dynamics of transmission of an amyloid proteinopathy has not been addressed yet in bacteria. Using time-lapse microscopy and a microfluidic set-up, we have assessed in *Escherichia coli* the vertical transmission of the amyloidosis caused by the synthetic bacterial model prionoid RepA-WH1 at single cell resolution within their lineage context. We identify *in vivo* the co-existence of two strain-like types of amyloid aggregates within a genetically identical population and a controlled homogeneous environment. The amyloids are either toxic globular particles or single comet-shaped aggregates that split during cytokinesis and exhibit milder toxicity. Both segregate and propagate in sub-lineages, yet show interconversion. ClpB (Hsp104) chaperone, key for spreading of yeast prions, has no effect on the dynamics of the two RepA-WH1 aggregates. However, the propagation of the comet-like species is DnaK (Hsp70)-dependent. The bacterial RepA-WH1 prionoid thus provides key qualitative and quantitative clues on the biology of intracellular amyloid proteinopathies.

Introduction

The amyloid cross-β assembly is the most stable thermodynamic state for a protein (Chiti and Dobson, 2006; Toyama and Weissman, 2011; Eisenberg and Jucker, 2012), whereas its native fold is metastable and kinetically favoured by natural selection to achieve a particular function (Baldwin *et al*., 2011). The amyloid aggregated state of some human proteins is at the basis of severe neurological disorders (e.g.: β-amyloid peptides in Alzheimer, α-synuclein in Parkinson, poly-Q repeats proteins in Huntington and spinocerebellar ataxias, SOD1 in amyotrophic lateral sclerosis), systemic diseases (such as β2-microglobulin in dialysis-related amyloidosis, amylin in type II diabetes and CryAB-desmin oligomers in heart hypertrophy) (Chiti and Dobson, 2006; Willis and Patterson, 2013). Amyloid aggregates behave as epigenetic determinants of traits inherited through non-Mendelian segregation. They propagate 'vertically' from mother to daughter cells where, as seeds, they template the amyloid conformation on soluble molecules of the same protein (Eichner and Radford, 2011). Prions are a subgroup of amyloids that are also 'horizontally' transmissible (infectious) (Tuite and Serio, 2010). They are pathogenic in mammals, such as PrP in transmissible spongiform encephalopathies (Cobb and Surewicz, 2009), but may confer selectable advantageous phenotypes to yeast (Halfmann *et al*., 2012).

Studies on prions provided evidence for the existence of strains, variants of the same polypeptide chain with slightly different three-dimensional structures that result in distinct phenotypes (Colby and Prusiner, 2011; Toyama and Weissman, 2011; Weissmann *et al*., 2011). Such structural polymorphism fuelled the quest for ligands, including chaperone macromolecules, modulating protein amyloidogenesis. Protein chaperones assist in the refolding and solubilization of aggregated proteins, as well as stabilize folding intermediates in the assembly pathways of macromolecular complexes, thus being central players in protein quality triage (Lindner and Demarez, 2009; Mayer, 2010; Tyedmers *et al*., 2010a). Two sets of ATP-dependent chaperones, the Hsp70-Hsp40-NEF (nucleotide exchange factor) triad and the Hsp104 hexameric disaggregase, act in concert disentangling large aggregates, including amyloids, into oligomers and soluble protein monomers (Glover and Lindquist, 1998; Goloubinoff *et al*., 1999; Walter *et al*., 2011; Winkler *et al*., 2012a,b). In yeast, Hsp104 can disrupt by itself the amyloid fibres of the Sup35/[*PSI*$^{+}$] prion, generating transmissible seed propagons (Shorter and Lindquist, 2004, 2006; DeSantis *et al*., 2012; DeSantis and Shorter, 2012), although it works more efficiently together with Hsp70 and Hsp40 co-chaperones (Shorter and Lindquist, 2008; Newnam *et al*., 2011). It is striking that no *HSP104* homologue has been found in metazoa, which instead resort to Hsp70-Hsp40-Hsp110 (Shorter, 2011; Winkler *et al*., 2012b; Rampelt *et al*., 2012).

The capacity to study proliferation of individual cells *in vivo* is limited by the number of generations that can be followed in real-time microscopy, as over-growth results in multi-layered micro-colonies that extend beyond the observation field (Stewart *et al*., 2005; Ackermann, 2008).

Nonetheless, the quantitative analysis of phenotypes of single cell lineages led to the discovery of replicative *aging* in bacteria (Stewart *et al*., 2005), which is linked to protein quality control through the preferential inheritance by the aging cells of a single sub-polar protein aggregate (inclusion body, IB) (Lindner *et al*., 2008; Winkler *et al*., 2010). Assurance of a homogeneous environment for cellular growth is another limitation of the standard agar pad support, so changes in the environmental conditions can be alleviated through its coupling with a manufactured microfluidic chamber (Balaban *et al*., 2004). Quantitative study of individual bacterial lineages for extensive number of generations (>200) has recently become amenable thanks to sub-micron resolution etching and the design of microfluidic devices (Vyawahare *et al*., 2010). 'Mother cell machines' are microfluidic devices particularly suitable for studying bacterial proliferation. They are made of narrow dead-end channels where a founder cell stays at the bottom of a micrometer-wide channel, while the daughter cells are pushed towards a perpendicular nutrient-supplying flow channel and are washed away (Wang *et al*., 2010).

While yeast serves as eukaryotic model system to untangle amyloidosis of clinical relevance, the continuous changes in cell volume and limited number of divisions of a given mother yeast cell before undergoing senescence reduce its applicability in high throughput microfluidic assays (Charvin *et al*., 2010). The successful propagation in *E. coli* of chimaeras between the yeast prion [PSI^+] and GFP has recently opened a heterologous approach, but it requires the help of yeast co-factors, such as seeds of the [PIN^+] prion (Garrity *et al*., 2010). Therefore, the study through microfluidics of the long-term vertical transmission of an amyloidosis using a fully bacterial model system would be of utmost interest.

We have recently reported that the WH1 domain of the bacterial plasmid replication protein RepA (Giraldo and Fernández-Tresguerres, 2004; Giraldo *et al*., 2003) is converted from its soluble, dimeric native structure to an amyloid state through a conformational change exerted by transient binding to short, specific DNA sequences *in vitro* (Díaz-López *et al*., 2003, 2006; Giraldo, 2007; Gasset-Rosa *et al*., 2008). When RepA-WH1 is expressed fused to a fluorescent protein in *Escherichia coli*, it aggregates as intracellular amyloid foci (Fernández-Tresguerres *et al*., 2010). Because RepA-WH1 amyloid aggregates are vertically transmissible within progeny, coupled to cell division (Fernández-Tresguerres *et al*., 2010) yet lack microbiological (horizontal) infectivity, RepA-WH1 can be considered as a 'prionoid' (Aguzzi, 2009). The RepA-WH1 prionoid causes a proteinopathy that severely reduces bacterial fitness (Fernández-Tresguerres *et al*., 2010), thus resembling closer mammalian rather than yeast amyloidosis. Overall, RepA-WH1 is a unique synthetic model system to study the molecular basis for protein amyloidosis in a minimalist cellular host (Giraldo *et al*., 2011).

In this article, we address, for the first time, the vertical transmission *in vivo* of an amyloidosis (the RepA-WH1 prionoid in *E. coli*) across multiple generations and at single-cell resolution using microfluidics. This revealed the existence of distinct populations of bacteria, bearing different species of RepA-WH1 aggregates with characteristic phenotypes: either a globular and highly cytotoxic form,

with multiple appearance per cell, or a single elongated, worm-shaped ('comet'-like) form mildly detrimental for cell proliferation. Both different aggregate species are of amyloid nature, thus being distinct variants ('strains'-like) of the prionoid. Interestingly, these two species are carried epigenetically through several generations and can dynamically interconvert. Since the DnaK (Hsp70) and ClpB (Hsp104) chaperones have a well-established role in disentangling protein aggregates in *E. coli*, we took full advantage of the robustness of the microfluidic setup to test the effect of these chaperones on RepA-WH1 amyloidosis, resorting to defective alleles, complementation assays and a small molecule inhibitor of DnaK, as well as to complementary biochemical and ultra-structural analyses. We found that the ClpB disaggregase has no major effect on amyloidosis, whereas DnaK specifically selects for, and enhances the inheritance of, the comet-shaped aggregates of the RepA-WH1 prionoid.

Results

The RepA-WH1(A31V) prionoid displays two inter-convertible, distinctly toxic aggregated forms

In order to characterize *in vivo* the vertical transmission of the bacterial prionoid RepA-WH1 (Giraldo *et al*., 2011) through tens of generations, we developed through high-resolution ion beam etching and double PDMS replica (Fig. S1) a comb-shaped microfluidic manifold in which a single *E. coli* 'mother' cell settles in the bottom of each micro-channel (Wang *et al*., 2010). We used *E. coli* MG1655 as wild-type strain, chromosomally expressing a translational fusion of the hyper-amyloidogenic variant His_6-RepA-WH1(A31V) and the mCherry (mRFP) fluorescent protein under the control of P*tac-lacI*^{q} (Fernández-Tresguerres *et al*., 2010) (Fig. S2).

Cells from a single clone were grown to exponential phase and then loaded in the microfluidic chip. After an adaptation period (about four generations), RepA-WH1(A31V)-mRFP expression was induced, leading to the appearance of two morphologically distinct aggregates, besides diffuse fluorescence: globular particles (Fernández-Tresguerres *et al*., 2010), distributed in various numbers across the cytoplasm, and 'comet'-shaped forms (Fig. 1AB; Movie S1). Image analysis of time-lapse movies was used to segment the cells within phase contrast images and classify the fluorescent aggregate phenotype within each cell (Fig. S3). This allowed to establish lineage relationship between phenotypes at each generation and to quantify the division time and growth rate for every cell. At any given time point, cells harbouring uniquely the globular form (characteristically 1-4 foci per cell) were significantly more abundant (40+/-5%; averaged over 10 divisions of 13 independent lineages, N=251) within the population as compared to cells solely carrying the comet-like form (25+/-3%). Cells bearing both forms were 26+/-3%, whereas 9+/-1% of the cells had no detectable aggregate. The globular aggregates grew from small foci (Movie S1) until they reached the full cell width dimensions (e.g.: Fig. 1B, top and bottom panels). The comet-shaped species often appear to originate and separate from a basal focus in the progenitor cells (e.g.: Fig. 1B, bottom panel; Movie S1). Upon

division, comet-shaped assemblies may split between dividing cells without imposing any apparent hindrance to mid-cell constriction, hence propagating in both offspring cells (e.g.: Fig. 1B, third panel). However, although cells that are born without any detectable aggregate (N=164) can get a globular focus before dividing again (37%), there is still a significant fraction of comet-only cells (17%) and a smaller fraction exhibiting both phenotypes (3%), the rest remaining void of aggregates until division.

In general, presence of any of the aggregate forms resulted in a significant increase in division time, indicative of a net reduction of cell fitness attributable to the toxicity of the RepA-WH1(A31V)-mRFP aggregates (Fig. S3). Cells carrying comet-shaped or globular foci had division rates significantly lower (1.5 hr^{-1}) than cells that had lost the aggregates through division (2.0 hr^{-1}; t-test $p<0.001$), or as compared to wild-type cells devoid of the prionoid construct (2.4 hr^{-1}; t-test $p<0.001$). Notably, cells with no fluorescent aggregates visible divided slower than those not expressing the prionoid, suggesting that some protein oligomers with sizes beyond the diffraction limit of conventional optical microscopy were present in the former, where they could eventually seed aggregation. On the other hand, cells accumulating 4 or more globular foci filamented frequently, exhibited much slower division (0.9 hr^{-1}) and, ultimately, stopped their growth and died.

Both RepA-WH1(A31V) aggregate forms propagate within bacterial lineages

In all cases, the progenitor ('mother') cell at the dead-end of each channel harboured a globular aggregate within 5 generations (Fig. 1; Movie S1). To assess the propagation of this mother cell-borne globular aggregate, we quantified the phenotype of all the immediate offspring of the mother cells across 30 generations (N=252). 40% of the progeny developed a new globular aggregate, 25% a comet-like aggregate, 26% bore both globular and comet-like aggregates and only 9% was voided of aggregates. This revealed the higher probability of transmitting the globular phenotype (66%) as compared to switching to the comet-like phenotype (51%). Overall the mother cells efficiently transmit the prionoid (with a probability close to 90%).

We then surveyed the persistence of both aggregate types by assessing the phenotype of all cells for which the progenitor was known and for which we could follow their offspring until division. For these cells we calculated the relevant transition probabilities, namely what was the aggregate phenotype distribution (Ø – no aggregate, G – globular, C – comet-like and GC – mixed phenotype), in the progenitors and offspring (Fig. 2). Given that each cell generates two, a probability >50% is indicative of propagation of a phenotype. A clear epigenetic memory emerged, whereby the aggregation phenotype in cells was clearly biased by their ancestor phenotype. Cells harbouring a globular focus were derived almost exclusively (>90%) from a G-type progenitor cell and most likely also gave G-offspring (80%). This also held, albeit to a lesser degree, for the comet-like species: >80% of their progenitor cells carried C-type aggregates and close to 70% of their offspring were C-phenotype cells (either pure C or GC). On the contrary, GC-phenotype was unstable (38% inheritance

per generation), segregating the G (34%) and C (25%) phenotypes. Notably, C-phenotype cells led to offspring with significant proportion of G-aggregates (23% pure G, 44% G+GC), suggesting that comet aggregates are relatively labile and can switch back to nucleate the more stable globular state. The reverse conversion (G to C-aggregates) was comparatively much disfavoured (9% pure C, 32% C+GC). Overall the emerging transition matrix is coherent with the steady-state proportions found in the population (*see above*): most cells (≈90%) acquired an aggregate within their life times with prevalence of the G- over the C- and GC-phenotypes. For the minority of cells (≈10%) that did not develop an observable aggregate before dividing again, only 19% of their offspring remained clear of aggregates, whereas the rest exhibited a clear bias towards the G-phenotype (44% pure G, 61% G+GC). As noted above on cell division rates, this observation suggests that the Ø-phenotype progenitor cells might carry small prionoid particles, beyond the resolution limits of conventional fluorescence microscopy, which once passed to the progeny will nucleate the growth of visible G-aggregates.

The RepA-WH1(A31V) globular and comet-like aggregates are amyloids

In order to check whether the two RepA-WH1(A31V) aggregate types (globular and comet-like) were of amyloid nature, fixed cells were incubated with BTA-1, a fluorophore specific for amyloid cross-β (Klunk *et al*., 2001). Both aggregate forms were positively stained (Fig. 3A), indicative of their amyloid character. As a control, in bacteria expressing RepA-WH1(ΔN37) a metastable mutant deprived of the amyloidogenic peptide stretch (Giraldo *et al*., 1998), either single or two sub-polar foci appeared in most cells (Fig. 1C; Movie S2), as expected for conventional IBs (Lindner *et al*., 2008; Winkler *et al*., 2010), yet no comet-like aggregates were observed. IBs include in their heterogeneous composition a nucleus with amyloid-like nature (Carrió *et al*., 2005; Wang *et al*., 2008). Indeed RepA-WH1(ΔN37) particles were also stained by BTA-1 (Fig. 3B). Quantitative analysis of BTA-1 staining (Fig. 3C) indicated a gradation in the amyloidosis of the distinct aggregates: globular WH1(A31V) > comet-like WH1(A31V) >> IB WH1(ΔN37).

The small heat-shock chaperone IbpA is a marker of *bona fide* IBs, where it targets aggregated proteins for refolding (Ratajczak *et al*., 2009). In cells in which an *ibpA-YFP* translational fusion was transduced (Fig. S2), yellow fluorescence co-localized with the sub-polar globular RepA-WH1(A31V) foci, but not with those in other locations across the cytoplasm or with the comet-like aggregates (Fig. S4; Movie S3). This result underlines that RepA-WH1(A31V) aggregates are not conventional IBs.

Because the globular and comet-like RepA-WH1(A31V) aggregates are phenotypically distinct (in terms of both morphology and relative toxicity) and are inherited across generations, being susceptible to spontaneous inter-conversion, they can be regarded as different variants of the same prionoid, resembling to some extent prion strains in mammals and yeast (Colby and Prusiner, 2011; Toyama and Weissman, 2011; Weissmann *et al*., 2011). However, the stability of the RepA-WH1 prionoid is much

lower than those for prions in eukaryotes, e.g. yeast, where rates around 10^{-6} for spontaneous phenotype loss or gain are typical (Halfmann *et al*., 2012).

The Hsp70 chaperone DnaK contributes to transmission and phenotype conversion of RepA-WH1 prionoid

We tested the possible role of the *E. coli* Hsp70 chaperone DnaK (Mayer, 2010; Tyedmers *et al*., 2010a) in propagation of the RepA-WH1 prionoid with myricetin, a specific inhibitor of the ATPase activity of DnaK by interfering with its regulation by DnaJ (Chang *et al*., 2011) (Fig. 4A; Movie S4). Most bacterial cells exhibited the globular aggregates, suggesting that DnaK is involved in the generation of the comet-like RepA-WH1 aggregates. Semidenaturing-detergent agarose gel electrophoresis (SDD-AGE) resolves any protein sample into its monomeric and oligomeric/aggregated fractions up to molecular weights well in the MDa range (Bagriantsev *et al*., 2006). Analysis by SDD-AGE of the intracellular RepA-WH1(A31V)-mRFP aggregates (Fig. 5A) showed that an oligomeric fraction was absent in the myricetin-treated cells (Fig. 4A). Myricetin has been reported to directly inhibit the amyloidosis of the Alzheimer's Aβ42 peptide (Ladiwala *et al*., 2011), hence we tested the effect of this polyphenol on RepA-WH1(A31V) amyloidosis *in vitro* (Fig. 5B): myricetin did not interfere with the assembly of the protein into oligomers and fibres. Given the null effect of this compound on RepA-WH1(A31V) amyloidogenesis *in vitro*, the lack of an oligomeric fraction *in vivo* (Fig. 5A), which correlates with the loss of the comet-shaped aggregates in the microfluidic assay (Fig. 4A; Fig. S5) can be attributed to inhibition by myricetin of DnaK (Chang *et al*., 2011). When DnaK was over-expressed from an arabinose (Ara)-inducible promoter cloned in a moderate copy-number (13-30 per cell) p15A plasmid replicon (Fig. S6B), cells in the channels either exclusively bore the comet-like aggregates or became apparently freed from aggregates (Fig. 4B; Movie S5). On the contrary, expression of the ClpB chaperone, whose Hsp104 homologue has a major role in propagation of yeast prions (Chernoff *et al*., 1995; Shorter and Lindquist, 2004, 2006, 2008), from a similar construct (Fig. S6B) did not alter the aforementioned proportions of the two RepA-WH1(A31V) aggregate forms (Fig. 4C; Movie S6). Overall, these results indicate that DnaK chaperone, rather than ClpB, is responsible for driving the balance between the globular and comet-like aggregated amyloid species of RepA-WH1(A31V) towards the latter.

In order to get further evidence on a role of DnaK chaperone in the dynamics of the RepA-WH1 prionoid, studies were also carried out with cells grown in bulk cultures. We had previously shown (Fernández-Tresguerres *et al*., 2010) that over-expression of His_6-RepA-WH1(A31V)-mRFP from a high copy-number plasmid (up to 250 copies/cell) led to the formation of globular amyloid inclusions, but not comet-like amyloids as those described here. We hypothesised that the high over-expression in that system, rather than the strain background, was causal to the exclusive selection of the globular phenotype. To this end, a low copy-number RK2 vector (5-8 copies/cell) was used to express His_6-RepA-WH1(A31V)-mRFP under a P*tac-lacI*^{q} cassette in the *E. coli* MC4100 strain and its isogenic

JGT20 (*dnaK756*) and JGT3 (Δ*clpB*) derivatives (Thomas and Baneyx, 1998) (Fig. S6A). Unlike a Δ*dnaK* mutant, the *dnaK756(ts)* allele allows for robust cell growth at the semi-permissive temperature at which the assays were performed (37 ^{o}C), yet exhibits impaired chaperone activity in protein refolding *in vitro* (Buchberger *et al*., 1999) and in assisting DNA replication, both *in vitro* and *in vivo* (Giraldo-Suárez *et al*., 1993).

Under these conditions, the expression of RepA-WH1(A31V) resulted in the accumulation, evident 30 min after the addition of the IPTG inducer, of fluorescent foci with heterogeneous numbers and distributions, essentially identical in the three strains tested (Fig. S7) and alike those observed in microfluidics (Fig. 1), or when expressed from higher plasmid copy numbers (Fernández-Tresguerres *et al*., 2010). The expression of RepA-WH1 in the *dnaK756* background, but not in the Δ*clpB* strain, led to a progressive loss of aggregates in the population (40 and 50% foci-free cells after 2 and 4 h, respectively; Fig. S8A). Since ribosome biogenesis is dependent on DnaK (Alix and Guérin, 1993) it is not surprising that the global expression level achieved for RepA-WH1 in the *dnak756* background was about 60% of that found in the two other strains (Fig. S6A). However, the lower RepA-WH1 levels in *dnaK756* must not be the cause of the drop in the number of cells carrying aggregates, because even lower levels were expected when the same expression cassette was inserted in the MG1655 chromosome as a single copy, and yet most of the cells bore foci (Figs. 1-3).

We then attempted complementation in the chaperone-deficient strains by expressing the *dnaK* or *clpB* genes (Fig. S9) cloned in the same compatible vector used in microfluidics (Fig. S6B). IPTG and Ara concentrations were calibrated through quantitative Western-blotting of the His_6 tag located at the N-termini of the three proteins. An equal expression of the RepA-WH1 prionoid and either chaperone was achieved during the first half of the experiment (0.5-2 h), and up to two-fold excess of chaperone molecules during the second half (2-4 h) (Fig. S6B). The order in the addition of each inducer was also tested at 30 min intervals, thus allowing for the accumulation of half the maximum level of the protein induced first before expressing the second one. This would reveal a possible effect of the chaperones on preventing protein aggregation (Ara added prior to IPTG) or on dismantling preformed RepA-WH1 aggregates (IPTG added prior to Ara). An increase in the average cell size and filamentation under heat-shock conditions are phenotypes characteristic of the *dnaK756* allele (Bredèche *et al*., 2001). Expression of DnaK reduced the average cell size by 40% (Figs. S9 and S8B) indicating successful complementation. Interestingly, the population of cells carrying aggregates increased to ≥90%, which is compatible with a role for DnaK in promoting the propagation of the RepA-WH1 prionoid. However, in the Δ*clpB* genetic background the fraction of cells bearing foci and the average cell size increased just slightly upon ClpB complementation (Fig. S8A,B), suggesting that both phenotypes are not dependent on this chaperone. There was no significant effect in MC4100 (WT) cells when either chaperone was expressed under the same conditions.

A major effect of DnaK complementation was a change in the appearance of the RepA-WH1 aggregates (Fig. S9), which in 70-95% of the cells (Fig. S8C) acquired the comet-like shape already

observed in the microfluidic setup upon DnaK overexpression (Fig. 4B). This suggests remodelling of the aggregates by the direct action of the Hsp70 chaperone. On the contrary, ClpB overexpression resulted in highly compacted foci (Fig. S9) in 75-95% of the cells, a fraction close to that observed in both *clpB*^{wt} and Δ*clpB* backgrounds (Fig. S8C) and in microfluidics (Fig. 4C). Regarding the number of aggregates per cell (Fig. S8D), the characteristic two foci (at ¼ and ¾ axial positions) (Fernández-Tresguerres *et al*., 2010) was the most abundant class (50-60%) in the MC4100 and Δ*clpB* cells. On the contrary, cells carrying multiple aggregates were most common in the *dnaK756* background, especially after 4 h of induction, reflecting the larger average cell size of this strain (Fig. S8B). The expression of either chaperone increased the proportion of cells with 3 or more aggregates to 50-70%. On the relative order of induction of prionoid and chaperones, expressing RepA-WH1 before ClpB made 1-2 aggregates per cell more common, whereas the opposite order of induction resulted in multiple aggregates as the dominant group. This suggests that high pre-existing levels of ClpB contributed to create multiple amyloid nuclei. In the case of DnaK complementation, cells with multiple aggregates were the most abundant class irrespective of the order of expression, as expected if the Hsp70 chaperone were more effective than the Hsp104 disaggregase in generating amyloid primordia.

DnaK remodels the RepA-WH1 aggregates and generates small oligomeric prionoid particles

To explore with further detail the action of DnaK and ClpB on the RepA-WH1 amyloid aggregates, we carried out immuno-localization studies with cells from bulk cultures expressing one of the two chaperones and the prionoid. Bacteria were visualized using confocal laser fluorescence microscopy (Fig. S10) and transmission electron microscopy (TEM) (Fig. 6). As expected from their large sizes, both the nucleoid and the RepA-WH1 particles mutually excluded. Neither DnaK nor ClpB colocalized with the nucleoid, whereas they targeted either the periphery of the globular aggregates (ClpB) or the tails of the comet-like aggregates (DnaK) (Fig. S10B). Immuno-electron microscopy (iEM) confirmed these distinct patterns and revealed that besides the large electron-dense RepA-WH1 globular aggregates (Fernández-Tresguerres *et al*., 2010) there were ellipsoid sectors, which probably reflect distinctive protein packing, where most of the DnaK label concentrated (labelled as C in Fig. 6A). Such elongated particles resemble the comet-like species of the RepA-WH1 prionoid seen in bacteria growing in the microfluidic channels (Figs. 1 and 4). In addition, some DnaK molecules also located on smaller particles (white arrows Fig. 6A), suggesting the generation of amyloid nuclei by DnaK-promoted fragmentation of the RepA-WH1 aggregates.

In order to survey a possible active role for DnaK in promoting disassembly of the prionoid, the purified chaperone was incubated, at physiological temperature and in the presence of its ATP cofactor, with RepA-WH1(A31V) amyloid fibres pre-assembled under standard conditions *in vitro* (Giraldo, 2007; Fernández-Tresguerres *et al*., 2010). DnaK was able to untangle the fibres into roughly homogeneous (≈20 nm Ø) globular particles that, according to iEM with anti-DnaK and anti-WH1

antibodies, were composed of both RepA-WH1 and chaperone molecules (Fig. 7). The anti-WH1 antibodies were also labelling the periphery of the fibres, suggesting that the epitopes must be either excluded from, or buried into, the axial core. However, DnaK was found only at the 20 nm particles, not on the fibres, suggesting that the chaperone, in its ATP form, must bind transiently to the latter, whereas the former must be the subsequent, disentangled and stable complexes between RepA-WH1 and DnaK-ADP (Mayer, 2010). Albeit this is purely an *in vitro* assay, it suggests that the oligomeric RepA-WH1 particles generated by DnaK action indeed have a suitable size for the effective diffusion of the prionoid across the cytoplasm to allow for its 'vertical' spread.

The distribution of RepA-WH1 between the soluble and aggregated fractions in whole cell extracts was analyzed by centrifugation and blotting with anti-His tag antibodies, which recognize RepA-WH1 and the DnaK/ClpB chaperones (Fig. S11A). As expected, most (≥60%) of the prionoid was found at the insoluble fraction. This was especially evident in the Hsp70-deficient (*dnaK756*) background (up to 80%; Fig. S11B). When either DnaK or ClpB were expressed, substantial amounts of the chaperones (50% at 2 h, and ≥75% at 4 h) were targeted to the aggregated fraction, whose contents in RepA-WH1 did not vary significantly. When total extracts from the *E. coli* cells were separated by SDD-AGE (Fig. 8), the existence of a major fraction of detergent-resistant RepA-WH1 oligomers became evident in the WT background. These oligomers completely disappeared in the *dnaK756* strain, while they reduced their presence by about 50% in the Δ*clpB* extracts (Fig. 8A). Complementation of *dnaK756* through the expression of DnaK led to the recovery of the detergent-resistant oligomeric RepA-WH1 fraction, whereas ClpB complementation had little effect (Fig. 8A). Although complexes between RepA-WH1 oligomers and DnaK were visualized by iEM (Fig. 6A), they seemed to be disrupted by the detergent during electrophoresis (Figure 8B), which was also the case for ClpB (Fig. 8C). The DnaK-dependent, detergent-resistant RepA-WH1 oligomeric species detected by SDD-AGE might correspond to the comet-shaped aggregated particles observed in the microfluidic experiments (Figs. 1 and 4) and through TEM (Fig. 6A).

The studies reported in this section, together with the evidence from microfluidics, point to a direct role of the Hsp70 chaperone DnaK in transforming large globular proteotoxic aggregates of the RepA-WH1 prionoid into a mixed pool of comet-like aggregates and relatively small oligomers, which readily diffuse to the offspring. These would be the bacterial counterparts of the transmissible amyloid propagons previously characterized in yeast prions (Tuite and Serio, 2010).

Discussion

One of the unsolved questions in protein science is the nature of the factors, besides mutations and extreme physicochemical conditions, that trigger the conformational changes enabling a folded soluble protein to self-assemble into amyloid aggregates *in vivo* (Giraldo, 2010). This conundrum is at the core of the biology of prions, a particular class of amyloidogenic proteins found in yeast and mammals

with the ability to act as infectious particles, ever since the proposal that a protein co-factor should be involved in facilitating the acquisition of the amyloid state by the PrP prion (Prusiner, 1998). A number of biomolecules, most of them polyanions, have been recently found to promote prion amyloidogenesis (Silva *et al*., 2008; Soto, 2011). However, molecular chaperones lead the race to become that missing cofactor for yeast prions, either by assisting the conformational conversion of native amyloidogenic proteins (Hsp70-40) or by actively catalyzing the shearing of amyloid fibres into small, and thus readily transmissible, particles (Hsp104) (Mayer, 2010; Tyedmers *et al*., 2010a). Many amyloidogenic proteins share with prions the ability to template the conversion of soluble protein molecules into the amyloid state and to be spread through 'vertical' (mother to daughter cells) transmission but, because they lack horizontal infectivity, they have been termed prionoids (Aguzzi, 2009). Prionoids include proteins involved in neurodegenerative diseases, in which Hsp70s have a protective role against amyloid aggregation (Muchowski *et al*., 2000; Luk *et al*., 2008; Hoshino *et al*., 2011). We have recently engineered a synthetic, minimal system to study protein amyloidosis, both *in vitro* and *in vivo*, based in the bacterial protein RepA-WH1 (Giraldo *et al*., 2011). RepA-WH1 behaves as a prionoid in *E. coli* (Fernández-Tresguerres *et al*., 2010) and shares with PrP having nucleic acids as co-factors promoting amyloidogenesis (Giraldo, 2007; Gasset-Rosa *et al*., 2008).

In this report we have characterized the vertical transmission of the RepA-WH1 prionoid in *E. coli* along tens of generations by using a microfluidic device coupled to time-lapse fluorescence microscopy. Microfluidics is an emergent approach to study biological macromolecules (Vyawahare *et al*., 2010) and individual cells (Yin and Marshall, 2012) in quantitative terms. A wealth of biologically sound information can be potentially extracted, upon altering physico-chemical variables and the composition of the buffer or culture media (including the presence of natural ligands, inhibitors or cofactors), on the behaviour of macromolecules or on cell physiology. In the device we adopted, single founder *E. coli* cells colonize the bottom of cell-wide microchannels that are continuously fed by culture medium under a laminar flow regime. Bacteria in the microchannels grow and divide with rates close to those in bulk liquid cultures (Wang *et al*., 2010), underlining the physiological relevance of the studies performed. The microfluidic setup has allowed us to survey the transmission dynamics of fluorescence-labelled RepA-WH1 within progeny cells, resulting from subsequent division rounds of a single mother cell, while they remain inside the microchannels (typically a propagating sub-lineage 'window' of 4-5 cells). The experiments presented here constitute the first application of microfluidics to the study of protein amyloidosis *in vivo*, adding to the recent precedent of the nucleation and growth on insulin amyloids *in vitro* (Knowles *et al*., 2011). At the whole organism level, prion amyloidosis is usually studied through genetic approaches in yeast (Alberti *et al*., 2013) and after successive passages through mice of infectious PrP particles, including or not intermediate cycles of amplification *in vitro* (Soto, 2011). In addition, heterologous propagation of a yeast prion between cultured mammalian cells has been addressed through fluorescence microscopy (Hofmann *et al*., 2013). However, the latter two approaches have the drawback of a low throughput. The feasibility

of bacteria in high-throughput screenings of amyloidosis has been recently proved by the development of a secretion assay in *E. coli* for extracellular amyloid assembly (Sivanathan and Hochschild, 2012). Beyond this, through microfluidics the minimal synthetic bacterial RepA-WH1 prionoid provides, at the single cell level and for lineages spanning many generations, a direct visual assessment of the existence and interconversion of two amyloid species resembling to some extent prion strains (Fig. 9): they are RepA-WH1 aggregates with characteristic morphology (globular *vs*. 'comet-like') and number (multiple *vs*. single) and different affinity for an amyloidotropic fluorophore and cytotoxicity (acute *vs*. mild). The existence of inter-convertible globular and elongated aggregates was also described for the yeast prion [*PSI*$^{+}$] (Tyedmers *et al*., 2010b; Kawai-Noma *et al*., 2010). While this paper was under review, the existence of small oligomeric species of [*PSI*$^{+}$] that, as those described here for the RepA-WH1 prionoid, enable inheritance in yeast but do not express the prion phenotype has been described (Dulle *et al*., 2013).

Combining microfluidics *in vivo* with complementary *in vitro* studies have also allowed us to address the question of how the Hsp70 (DnaK) and Hsp104 (ClpB) bacterial chaperones influence the propagation and structural dynamics of the RepA-WH1 prionoid. Sequential cooperation between Hsp70s and Hsp104 is instrumental in prion propagation in yeast (Shorter and Lindquist, 2008; Winkler *et al*., 2012b). In the case of RepA-WH1, when assessed individually, DnaK seems to have a major role in promoting prionoid propagation whereas ClpB has a marginal influence. We have found that in *E. coli* DnaK enables RepA-WH1 prionoid transmissibility by generating, from compact globular amyloid aggregates, small oligomeric particles (alike those generated by the chaperone *in vitro*) and comet-shaped structures that are readily propagated to the offspring cells (Fig. 9A). According to iEM, in the bacterial cells molecules of this Hsp70 chaperone cluster at defined elongated substructures within the RepA-WH1 amyloid aggregates, reminiscent of recent observations in yeast pointing to a possible role for members of the same chaperone family in holding together bundles of the [*PSI*$^{+}$] prion (Saibil *et al*., 2012). Under physiological conditions, the C-shaped aggregation state of the RepA-WH1 prionoid is readily achieved with the help of DnaK, but it can spontaneously reverse towards the G-type, suggesting that both represent close local, rather than global, energy minima (C > G) in the protein folding funnel (Eichner and Radford, 2011; Baldwin *et al*., 2011) (Fig. 9B). The feasibility of improving the segregational stability of the RepA-WH1 prionoid through directed molecular evolution procedures remains to be determined. Protein dimers of the Rep family of plasmid DNA replication initiators are remodelled by DnaK to become active monomers, through binding to their WH1 domains (Kim *et al*., 2002). Thus it makes sense that the same chaperone modulates the disassembly of the RepA-WH1 amyloid aggregates into discrete oligomers, as reported here. The observed inter-conversion between RepA-WH1 aggregate types assisted by Hsp70 parallels Hsp104-dependent selection of strong over weak [*PSI*$^{+}$] strains in yeast (DeSantis and Shorter, 2012). However, compared with the extraordinary mitotic stability of yeast prion strains, the two strain-like variants described here for the bacterial RepA-WH1 prionoid have

marginal segregational stabilities (Fig. 9C). Whether this meta-stability of the epigenetic phenotypes in the synthetic prionoid reflects a genuine structural feature of RepA-WH1 or it is idiosyncratic of the bacterial host (e.g., its protein quality control machinery) remains to be determined. It is noteworthy that the coexistence in single yeast cells of several prion variants along generations has been recently reported for [*PSI*$^{+}$], and interpreted as a stage in the conformational maturation of the distinct prion strains (Sharma and Liebman, 2012). It might be that, since it is synthetic and thus not naturally spread across bacterial populations, the RepA-WH1 prionoid still had no time to evolve towards the stable propagation of a mature conformation.

The bimodal (globular *vs*. comet-like) shape distribution of the aggregates, their high proteotoxicity, poor co-localization with IbpA and avidity for an amyloidotropic agent clearly set the RepA-WH1 prionoid apart from conventional IBs. It is noteworthy that DnaK was previously found to be distributed around the surface of IBs (Carrió and Villaverde, 2005) not, as described here for RepA-WH1, selectively clustered in defined sub-sectors inside the aggregates. It had been proposed that ClpB disassembles proteins that aggregate as IBs upon overexpression, in a pathway towards their solubilization and/or proteolytic degradation (Doyle and Wickner, 2009). For the RepA-WH1 prionoid, we have found in its independence on ClpB for propagation another difference with conventional IBs. Exhaustive biochemical studies on the role of Hsp104 chaperones in disaggregation of amyloid and non-amyloid (heat stress-induced) aggregates have recently shown that the probabilistic intersubunit cooperation mechanism in bacterial ClpB is unable to disentangle amyloid aggregates but it is operative on disordered aggregates. However, yeast Hsp104 can deal with amyloids through an additional mechanism that implies substrate-tailored modulation of intersubunit cooperativity (DeSantis *et al*., 2012). Our findings here on ClpB and RepA-WH1 are compatible with such scenario.

Considering the apparent absence of any *HSP104* gene in higher eukaryotes, the results reported here underline recent findings pointing to Hsp70s chaperones as modulators of protein amyloidosis in mammalian cells (Shorter, 2011; Winkler *et al*., 2012b; Rampelt *et al*., 2012) and support the proposal of altering Hsp70s expression and/or activity as a way to reduce amyloid toxicity (Donmez *et al*., 2012; Chafekar *et al*., 2012). Mitochondria, which are recurrently found implicated in the pathways for protein amyloidosis (Itoh et al. 2013), were once endosymbiotic bacteria. It follows that versatile and minimal synthetic bacterial model systems, as shown here for the RepA-WH1 prionoid in *E. coli*, can provide valuable insights on amyloid proteinopathies in mammals.

Experimental Procedures

Gene constructs

Chromosomal insertion of the *His6-RepA-WH1(A31V/ΔN37)-mCherry* expression cassettes are described in Fig. S2. pRK2-*WH1(A31V)-mCherry* and p15A-*dnaK*/*clpB* plasmid vectors were constructed as indicated in Fig. S6.

Microfluidics

Microfluidic cells were fabricated as described in Fig. S1. *E. coli* K12 MG1655 strain derivatives (Fig. S2) were grown overnight at 37 ºC in minimal medium supplemented with glucose and casamino acids (M9-CAA). Then 50 µL of each cell culture were diluted in 20 mL of fresh medium and were grown to an OD_{600nm}=0.2. Cultures were centrifuged, washed twice with M9-CAA supplemented with PEG4000 to 1.5% and the cell pellets were resuspended in 200 µL. Cells were then loaded in the microfluidic chip by a centrifugation pulse and the device was immediately placed at a fluorescence microscope (see below). A continuous (2 $mL.h^{-1}$) laminar flow of filtered M9-CAA at 37 ºC was passed through the setting by means of syringes coupled to inlet valves. When required, chloramphenicol (30 $\mu g.mL^{-1}$), myricetin (15 $\mu g.mL^{-1}$; Sigma), IPTG (0.05 mM) or L-arabinose (0.15%) were injected. Bacterial growth was followed for 10-72 h, taking each 6 min up to 15 image frames across the chip. Correlative TIFF images were edited as movies using ImageJ (Collins, 2007).

Bulk bacterial cultures

E. coli K12 MC4100 cells, or their isogenic strains JGT20 (*dnaK756*) and JGT3 (Δ*clpB*) (Thomas and Baneyx, 1998), were inoculated in LB medium supplemented with 0.1% glucose and, if required, with ampicillin (100 $\mu g.mL^{-1}$) and chloramphenicol (30 $\mu g.mL^{-1}$). Bacteria were grown at 37 ºC to exponential phase (OD_{600nm}=0.2) and then induced with 0.5 mM IPTG. 1 mL aliquots were taken every 30 min for 4 h. For dual chaperone and WH1 expression, the promoter-specific inducers (0.1 mM IPTG and 0.5% L-arabinose) were successively added within a 30 min interval.

Fluorescence microscopy

Observation of bacterial cells from bulk cultures was performed with a Nikon Eclipse 90i microscope, equipped with a CFI PLAN APO VC 100x (NA 1.40) oil immersion objective and a Hamamatsu ORCA-R^2 CCD camera. The following excitation (EX) and emission (EM) filters and exposure times were used: m-Cherry (EX 543/22, EM 593/40; 200 ms), BTA-1 (EX 438/24, EM 483/32; 3 s). A DIC image of each field was also captured (100 ms). Images were analyzed with the Nikon NIS-Elements AR software. Bacterial culture aliquots were fixed in formaldehyde and mounted on poly-L-lysine coated slides (Fernández-Tresguerres *et al*., 2010). Amyloid-specific staining with BTA-1 (2-(4´methylaminophenyl) benzothiazole; Sigma, 0.5 mM from a stock in DMSO) was performed for 20 min during fixation. Bacterial growth in the microfluidic cell was followed with a Nikon Eclipse Ti inverted microscope, equipped with a 100x objective and phase contrast optics, similar filters and coupled to a Photometrics Cool-Snap HQ2 CCD camera. Image acquisition at this microscope set was controlled by MetaMorph software (Molecular Devices). The following exposure times were used: 100 ms of transmitted light (ND32 neutral filter, for phase contrast), 1 s (mCherry) and 2 s (YFP).

Transmission electron microscopy (TEM)

RepA-WH1(A31V) amyloid fibres (Giraldo, 2007) were assembled *in vitro* using as seeds purified intracellular RepA-WH1(A31V)-mRFP aggregates (Fernández-Tresguerres *et al*., 2010). Fibres were then examined on carbon coated 400-mesh copper grids (Ted Pella, Inc.) after negative staining with 2% uranyl acetate. For immuno-electron microscopy (iEM), RepA-WH1(A31V) fibres were incubated with 25 µM DnaK in 0.1 M Na_2SO_4, 10 mM Mg_2SO_4, 20 mM HEPES pH 8.0, 2 mM β-MeEtOH, 10% glycerol, +/- 0.1 mM ATP, at 37 ºC for 1h. For immuno-electron microscopy (iEM) with *E. coli* cells, bacteria were processed as described (Fernández-Tresguerres *et al*., 2010). The primary antibodies used were either rabbit polyclonals, raised against purified RepA-WH1(A31V) (1:10) or ClpB (Tek and Zolkiewski, 2002; 1:5,000), or a mouse monoclonal anti-DnaK (clone 8E2/2, Enzo Life Sciences; 1:200). Anti-mouse or anti-rabbit antibodies conjugated with 10 nm Ø gold particles (Sigma; 1:50, for 1 h) were then used as secondary probes (Fernández-Tresguerres *et al*., 2010). When iEM was performed on fibres assembled *in vitro*, these were adsorbed on glow-discharged grids and successively floated on drops of 0.05% Tween-20 and 2% BSA in PBS for 30 min and the anti-DnaK or anti-WH1 primary antibodies (1:500, 1 h at room temperature). After three washes (10 min each) with 0.05% Tween-20 in PBS, grids were incubated with Au-conjugated secondary antibodies (1:50, for 1 h). Samples were then washed three times with Tween-PBS and once with bi-distilled water before uranyl acetate staining. Specimen observation was carried out in a JEOL JEM-1230 transmission electron microscope operating at 80 kV and images were captured with a TVIPS TemCam-F416 CMOS camera.

Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)

Cells from 25 mL cultures were harvested after 2.5 h induction (OD_{600nm} ≈ 2.0) and resuspended in 400 µL of 25 mM Tris.HCl pH 6.8, 250 mM NaCl, 5 mM EDTA, 10% glycerol, supplemented with protease inhibitors (Roche). 1/3 of the volume of this suspension was added to a tube containing silica beads (1.0 mm Ø, Lysing Matrix C, MP Biomedicals) and the mixture was shaken in a FastPrep-24 homogenizer (4 cycles at speed-IV, 30 s each, 4 ^{o}C). 3 µL of each lysate were diluted to 40 µL with 0.5xTAE, 5% glycerol, 2% sarkosyl, 0.1 $mg.mL^{-1}$ bromophenol blue, plus protease inhibitors. Samples were incubated at room temperature for 10 min and then loaded in 1.5% agarose-TAE, 0.1% SDS gels. Electrophoresis was run at 100 V for 7.5 h at 10 ºC. Gels were then electro-blotted (Transfer Blot, BioRad) to PVDF membranes in TAE-0.1% SDS at 16 V/400 mA for 15 h at 10 ^{o}C. After membrane blocking, primary antibodies were incubated for 2 h: anti-WH1(A31V) (1:500), anti-DnaK (1:2,500) or anti-CplB (1:3,000). Labelling with HRP-conjugated antibodies and detection were carried out as indicated in Fig. S5. Washing in 8 M Gu.HCl for 1 min allowed to strip membranes for successive antibody probing.

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Figure Legends

Figure 1. Selected time-elapsed frames showing *E. coli* cells in five of the channels (vertically aligned) in the microfluidic device. Mother cells occupy the bottom of each micro-channel, whereas fresh medium flows horizontally at the top.

A. Fluorescence emission of the RepA-WH1(A31V)-mCherry (mRFP) particles indicates the presence of two types of protein aggregates: either globular (labelled with red stars below the mother cells) or comet-shaped (yellow stars). In any lineage, generation of globular or comet-like aggregates can switch with low frequencies. Double coloured stars indicate cells lineages in which globular and comet aggregates coexist.

B. Magnifications of representative bacteria carrying globular or comet-like aggregates, as visualized through red (prionoid, mRFP) and yellow (cytoplasm, YFP) fluorescence emission or phase contrast (PhC).

C. A composition of microchannels with bacteria carrying RepA-WH1(ΔN37)-mCherry IBs. Cells in all channels carry 1-2 subpolar globular aggregates (red stars). For a full account, see Movies S1 (A) and S2 (C).

Figure 2. Frequencies of the different transitions between aggregate phenotypes observed during propagation of the RepA-WH1(A31V) prionoid in the microfluidics device. The four characteristic phenotypes are indicated at the centre of each quadrant (boldface): Ø – no aggregate, G – single globular focus, C – comet-shaped and GC – for mixed phenotype. Each one is coupled to the phenotype of its progenitor cell, as well as to its direct offspring cells (arrows). A subset of cells (N=1884) was chosen for the analysis, among the hundreds across the experiment, based on the criteria of the unambiguous identification of their progenitors and the feasibility of following their offspring until division. This excluded basal mother cells and those washed away from the channels. Solid brackets span numbers relevant for the epigenetic transmission of the two aggregate phenotypes, whereas grey dashes underline those related to phenotype switches.

Figure 3. The amyloid-specific fluorophore BTA-1 targets the RepA-WH1 intracellular aggregates.
A. DIC (*left* panels), and red (mCherry, *centre*) and cyan (BTA-1, *right*) fluorescence images of single cells bearing the globular (*top*) and the comet-like (*bottom*; arrows) aggregates.
B. Bacteria carrying RepA-WH1(ΔN37) IBs (see A).
C. Box-plot of the ratios between the fluorescence intensities of the cyan and red channels for each type of aggregate. Particles were picked-up from 25 images (16 bit grey-scale mode) in the two channels, acquired under constant optical settings (see Experimental Procedures). Calculations were performed with ImageJ, using elliptical sector probes (comprising 166 pixels) that were centred on 75 single, neat particles of each type. Dots mark the mean values, bars span the minimal and maximum intervals of dispersion, boxes comprise 25-75% of the data points and the thick horizontal lines indicate the median values. To test if there were any differences in the affinity of the distinct RepA-WH1 aggregates for BTA-1, the null hypothesis was contrasted by statistical analysis of the variance for particles belonging to the three types. One-way ANOVA statistical analysis ($F=98.31$, $p<0.0001$) followed by Tukey's pairwise difference test indicated that each kind of aggregate belongs to a distinct class in terms of BTA-1 staining.

Figure 4. Microfluidic channels showing bacteria expressing RepA-WH1(A31V)-mRFP in which the effect on the propagation of the prionoid of the Hsp70 and Hsp104 chaperones in *E. coli* was tested by means of treatment with the DnaK (Hsp70) inhibitor myricetin (**A**), or overexpression of either DnaK (**B**) or ClpB (**C**). While DnaK seems to have an effect on the interconversion between the globular (red stars) and the comet-like (yellow stars) aggregates, because the former accumulate upon DnaK inhibition (A) and the latter under DnaK overexpression (B), ClpB has no apparent role, since both

aggregated species coexist along the whole experiment. See also Movies S4 (A), S5 (B) and S6 (C). The molar ratios between each chaperone and RepA-WH1 were estimated to be between 0.5-2.0, based on Western-blot measurements performed on cells from bulk cultures under the expression conditions (<2 h after induction at OD_{600nm}=0.2) closest to those at the microfluidic device.

Figure 5. Effects of the polyphenol myricetin on RepA-WH1(A31V) amyloidosis, either *in vivo* (A) or *in vitro* (B).

A. Bacteria grown in the absence or in the presence of myricetin (35 μM) were harvested after 4 h induction and lysated, and semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) carried-out, as indicated in Experimental Procedures (see also Fig. 8). Anti-WH1 primary polyclonal antiserum (1:2,000) and a secondary anti-rabbit HRP-conjugated antibody (1:50,000) were used for immuno-detection of RepA-WH1(A31V). A control of monomeric RepA-WH1(A31V) not-fused to mRFP was included by boiling a sample of the purified protein immediately before loading the gel. SDD-AGE indicates that the fraction of SDS-resistant RepA-WH1(A31V)-mRFP amyloid oligomers disappears upon treatment of the cells with myricetin.

B. RepA-WH1(A31V) fibrillation assays were performed as described elsewhere (Giraldo, 2007; Fernandez-Tresguerres *et al*., 2010) in the presence of the indicated concentrations of myricetin. Unlike it had been reported for the amyloid-β peptide (Ladiwala *et al*., 2011), no difference was observed in the assembly of the protein fibres that could be attributable to myricetin.

Figure 6. Intracellular distribution of DnaK and ClpB chaperones and RepA-WH1(A31V)-mRFP revealed by iEM. Thin sections of *E. coli* cells bearing the RepA-WH1(A31V) aggregates and complemented with DnaK (**A**) or ClpB (**B**) were incubated with specific antibodies against these chaperones and then localized with Au-conjugated antibodies (dots). N: nucleoid. The electron-dense areas are the RepA-WH1(A31V) aggregates (Fernández-Tresguerres *et al*., 2010), either globular (labelled as G) or comet-shaped (labelled C). The G-aggregates do not exhibit significant DnaK binding, whereas the elongated C-particles and a few smaller foci (arrows) concentrate most of the anti-DnaK labelling. On the contrary, ClpB binds to the aggregates evenly.

Figure 7. RepA-WH1(A31V) amyloid fibres assembled *in vitro* (Giraldo, 2007; Fernández-Tresguerres *et al*., 2010) were incubated with purified DnaK and ATP, resulting in disorganization of the fibres by the action of the Hsp70 chaperone. Au-conjugated secondary antibodies label (dots) the location of primary antibodies against RepA-WH1 (α-WH1) or DnaK (α-DnaK). Whereas α-WH1 decorates both the surface of the fibre scaffold (its axis highlighted with a dotted line) and the small (≈20 nm Ø) spherical particles detached from the fibres, α-DnaK mostly labels the latter.

Figure 8. Biochemical characterization of the presence of RepA-WH1(A31V)-mRFP oligomers in *E. coli* and their dependence on DnaK/ClpB chaperones.

Whole cell extracts of the WT and DnaK/ClpB-deficient strains, complemented or not by the expression of these chaperones (the arrow labelling, Amyl → DnaK/ClpB or DnaK/ClpB → Amyl, indicates the sequential expression of the prionoid and the chaperones; Figs. S9 and S10), were analyzed by SDD-AGE. The distribution of RepA-WH1(A31V)-mRFP (**A**), DnaK (**B**) and ClpB (**C**) was revealed by means of Western blotting with specific antibodies. The presence of detergent-resistant RepA-WH1(A31V) oligomers was strictly dependent on the Hsp70 chaperone, but not on the Hsp104 disaggregase.

Figure 9. Cartoon models for the generation (A), folding/aggregation landscape (B) and propagation (C) of the RepA-WH1 prionoid in *E. coli*. Co-factors are depicted in green (DNA effector) and blue (Hsp70 chaperone DnaK). Figure elements not drawn to scale.

A. A mechanistic, sequential view of RepA-WH1 amyloidogenesis. Upon allosteric binding to DNA at the bacterial nucleoid, with a preference for specific sequences (*opsp*; Fernández-Tresguerres *et al*., 2010), the native dimers of RepA-WH1 (dWH1) undergo a conformational change to a β-sheet-enriched monomeric conformation (mWH1*) (Giraldo *et al*., 2003), which is aggregation-prone. The resulting oligomeric amyloid aggregates ($mWH1_n$; amyloid conformers depicted in red) act as seeds that enable the growth of larger aggregates by templating the amyloid conformation on native dWH1 molecules (Giraldo, 2007). Mature RepA-WH1 amyloids appear as two distinct species, either globular (G) or comet-shaped (C) aggregates, having DnaK a key role in the generation of the C-type either by assisting in amyloidogenesis or by remodelling the globular aggregates.

B. RepA-WH1 amyloidogenesis in terms of the free energy (ΔG) of folding/aggregation (Eichner and Radford, 2011). Folded from a newly synthesized molten monomeric precursor (M), stable native dWH1 populates a higher energy metastable state (mWH1*) upon conformational selection exerted by the DNA allosteric effector. Such metastable species assembles into forms with increasing stabilities/lower free energies: $(mWH1)_n$ oligomers → C-aggregates → G-aggregates. The DnaK chaperone would act either by stabilizing the (relatively) high energy C-type aggregate, or by generating C as an intermediate in disassembly of the lowest energy G-type aggregate into oligomers.

C. A view on the epigenetic transmission of the RepA-WH1 prionoid. *Top row*: the microfluidic analysis of the aggregation dynamics of the RepA-WH1 prionoid in *E. coli* cell lineages reveal that its two amyloid phenotypes (G and C) can interconvert through intermediate cellular states carrying both globular and comet-shaped aggregates (GC), but the G phenotype dominates displacing the equilibrium leftwards. The reverse switch is promoted by the DnaK chaperone following the sequence: G → GC → C. *Bottom row*: the phenotypes in the progeny are summarized by showing the three most representative types of descendants (those found >10%) from each G, GC or C parental cell, with the thickness of the dotted lines being roughly proportional to the frequency of each offspring class (for a

quantitative analysis, see Fig. 2). The most toxic species (G) results in the filamentation, proliferation arrest and, ultimately, death of a substantial fraction of the cells within its lineage. The mildly toxic species (C) propagates with a lower burden on cell fitness. For both kinds of aggregates, the phenotype tends to be held in the progeny, as expected for epigenetic inheritance.