SUPPORTING INFORMATION

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

Fátima Gasset-Rosa, Anne-Sophie Coquel, Maria Moreno-del Álamo, Peipei Chen, Xiaohu Song, Ana M. Serrano, M. Elena Fernández-Tresguerres, Susana Moreno-Díaz de la Espina, Ariel B. Lindner and Rafael Giraldo

1. Department of Cellular & Molecular Biology, Centro de Investigaciones Biológicas – CSIC, C/ Ramiro de Maeztu 9, E-28040 Madrid, Spain.
2. Center for Research and Interdisciplinarity (CRI), Faculty of Medicine, Descartes University – INSERM, 24 Rue du Faubourg St Jacques, F-75014 Paris, France.

* Corresponding authors. E-mails: ariel.lindner@inserm.fr; rgiraldo@cib.csic.es
The fabrication of the chips consisted in three main stages (A, columns). Firstly, we made a mold with the pattern on a silicon substrate. The growth channels were firstly patterned with poly-methylmethacrylate (PMMA) using electron-beam lithography: we spin-coated a 2.3 µm thick layer of PMMA (950K A11 positive electron-beam resist) onto a silicon substrate at 3,000 rpm for 1 min and bake it at 175 °C for 10 min (step 1). Then we used a Leica EBPG 5000+ with 100 kV accelerating voltage and 400 µm of aperture to script the growth channels in it (step 2). The conditions were 95 nm spot size, 188 nA beam current, 100 nm beam step size (BSS) and a 1300 µC/cm² dose. Following electron-beam exposure, we developed the exposed PMMA by using methyl-isobutyl ketone (MIBK) and isopropyl alcohol (IPA), at a 3:1 ratio for 60 s, and then IPA for 30 s (step 3). After spinning, dry etching was done for 3 min using an inductive coupled plasma (ICP) reactive-ion etcher (RIE) (Adixen AMS 100 I-speeder) with a mixture of 15 sccm sulfur hexafluoride (SF₆), 20 sccm octafluorocyclobutane (C₄F₈), 10 sccm methane (CH₄) and 100 sccm helium (He) (step 4). After cleaning (step 5), we fabricated the main channels on silicon. The procedure for writing main channels on PMMA (steps 6-8) was identical to the steps 1-3 above. We then utilized a Bosch deep reactive ion etching (DRIE) process to engrave the main channel. Etching was performed by 200 sccm SF₆ for 7 s with 2000 W ICP power with subsequent passivation by 80 sccm C₄F₈ for 2 s. We repeated these cycles for 2 min. After cleaning (step 9), the silicon mold with both growth and main channels was ready (B, left and middle panels). Secondly, we transferred the patterns to polydimethylsiloxane (PDMS) surface by double replication. PDMS (10:1 weight ratio) was poured onto the silicon mold and baked at 75 °C for 2 h, and then was peeled-off to get the negative structure on PDMS as the second mold (step 10). Before replication of patterns from this mold, trimethylchlorosilane (TMCS) was self-assembled on the surface to reduce the adhesion between contiguous PDMS layers. After that, PDMS was poured onto the mold and baked at 75 °C for 2h. By peeling-off the two layers (step 11), we got the same patterns with the silicon mold on the PDMS slab (B, right panel). Finally, chip sealing was performed using this PDMS slab and a glass slide (step 12). After drilling holes at the inlet and outlet of the chip, this was mounted onto a clear glass slide (with patterns facing the glass slide) and baked at 75 °C overnight to get the integrated microfluidic chip (step 13).
Figure S2. Scheme on the construction of Escherichia coli K-12 MG1655 strain (Blattner et al., 1997) derivatives carrying a His$_6$-RepA-WH1(A31V/ΔN37)-mCherry $P_{tac}/lacI$ expression cassette inserted in the chromosome. (A) The RepA-WH1(A31V) and ΔN37 expression modules were obtained by SpeI and EcoRI digestion on the pSEVA121 (RK2)-derivatives described in Fig. S6A. DNA fragments were then cloned into pSEVA511, a tetracycline-resistant (Tc$^R$) vector carrying the origin of replication of the plasmid R6K (ori$^\gamma$), but lacking the gene coding for its replication initiator protein (pir), sharing with pSEVA211 the same multicloning restriction sites (http://seva.cnb.csic.es/SEVA; Silva-Rocha et al., 2013). This plasmid can be maintained in the E. coli strain DH5α-λpir, which carries a chromosomal insertion on the pir gene. The resulting pR6K derivative was then transformed by electroporation into the strain MG1655 and Tc selection recovered clones lacking free plasmids but in which the whole pR6K construct had been inserted into the chromosome of the recipient cells, as confirmed by PCR amplification using the oligos 5’GCTACTAGTGGACAATTAAATCATCGGCTCG (Ptac-SpeI) and 5’AGTGAATTCGAGCTCGTAC (Ptac-EcoRI), bearing the indicated recognition sites (underlined). The expression of the RepA-WH1-mCherry fusions was verified under the fluorescence microscope. (B) The resulting strains were transduced (left) by inoculation with stock supernatants of cell cultures previously infected with P1 bacteriophages, carrying a Cm$^R$ marker and either the YFP gene (to label the cytoplasm) or an IbpA-YFP fusion (Lindner et al., 2008), or transformed (right) with p15A-derived (Cm$^R$) Para expression vectors with the chaperone genes dnaK or clpB cloned (see Fig. S6B).
Figure S3. Quantification of the effect of the RepA-WH1(A31V) prionoid on the cell division of *E. coli* MG1655. Histogram bars (cells bearing): 1C, a single comet-like aggregate; nG, n globular aggregates; 4G+, four or more globular aggregates; GC, a single globular aggregate plus a comet (cells undergoing a phenotype switch); No, no aggregate evident; MG, no RepA-WH1 expressed (MG1655 negative control). The number of cells counted and the statistical significance (**: p<0.001) in a Student's t-test are indicated. MG1655 cells grow faster than any of the other phenotypes, including cells with no visible aggregates. Cells with no visible aggregates grow faster than cells with aggregates. Bacteria with 4G+ phenotype grow slower than cells with 1C or 1-3G. Cells with GC phenotype grow slower than those with 1C or 1-3G.

Data analysis was carried out with a plugin to ImageJ developed at INSERM U1001. For each channel, frames were created separately for the phase contrast and the fluorescence images as a function of time. A low pass filter (3x3 pixels) was applied to these frames and then images were normalized by background subtraction. Fluorescence images were stabilized (Image Stabilizer) and phase contrast images were de-noised using a total variation algorithm (Rudin *et al.*, 1992). Cells were segmented automatically using threshold and local histogram methods, with final manual adjustment. The lineage from each mother cell was based on a morphological parameter: upon division, the total length of the two daughter cells must be over 90% that of the ancestor cell. To follow protein aggregates, fluorescence and phase contrast images were overlapped. The ImageJ plugin implements a threshold based on circularity to distinguish between globular and comet-like aggregates, although some manual corrections were still necessary for segmentation of both cells and aggregates. All dividing cells were classified on the basis of the number and type of aggregates by an R-script.
Figure S4. Tracing by microfluidics the presence of IB-like aggregates in bacteria propagating the RepA-WH1(A31V)-mRFP prionoid. The figure shows snapshots of five micro-channels across the time, with parallel displays of mRFP (red channel) (A), IbpA-YFP (Lindner et al., 2008; displayed in green, rather than in yellow, to enhance contrast) (B), and their overlay (C). The switching between the globular and comet-like particles characteristic of the prionoid is indicated in C as red or yellow stars, respectively. IbpA, a marker of IBs, colocalizes with some of the globular aggregates of the prionoid when these are in subpolar position, but not if located elsewhere or with the comet-like aggregates. For a full account, see Movie S3.
Figure S5. Frequencies of the different transitions between aggregate phenotypes observed during propagation of the RepA-WH1(A31V) prionoid in the microfluidics device, either in the presence of the DnaK inhibitor myricetin (left) and upon overexpression of DnaK (right). Data were extracted from Movie S4 (+ myricetin) and Movie S5 (+ DnaK), implying the analysis of 75 and 1109 cells, respectively. These results illustrate a strong bias towards the generation of cells bearing G-aggregates in the presence of the DnaK inhibitor and, in a less acute way, towards the C-phenotype in the case of DnaK overexpression, compared with the fractional probabilities reported for the A31V control (Fig. 2). However, the overexpression of the Hsp70 chaperone increases significantly the fraction of cells bearing no visible aggregate, which actually becomes the most frequent phenotype. Whether this reflects the generation of very small particles, beyond the resolution limit for their detection, and/or curing of the aggregation phenotype remains to be determined.
Figure S6. Vectors used to express the A31V and the ΔN37 variants of the RepA-WH1-mRFP prionoid (A) and the DnaK or ClpB chaperones (B). pRK2-WH1(A31V)-mRFP was built from pWH1(A31V)-mRFP (Fernández-Tresguerres et al., 2010) by PCR amplification of its whole P_tac expression cassette using Pfu DNApol and the primers P_tac-SpeI and P_tac-EcoRI (see Fig. S2A). The SpeI and EcoRI-digested fragment was cloned into the low-copy number vector pSEVA121 (RK2 mini-replicon, Ap<sup>R</sup>; http://seva.cnb.csic.es/SEVA; Silva-Rocha et al., 2013). The resulting plasmid was then digested with KpnI and EcoRI and these sites were used to clone lacI<sub>q</sub>, amplified by PCR on the pET11a vector (Novagen) with the following primers: 5′GCTCGAGGTCACCGCCTCTACGCCGGAC (lacI<sub>q</sub>-KpnI) and 5′CGAGCGCCGTTACTGGACGGCGACAATCCG (lacI<sub>q</sub>-XmaI). PCR products were digested with the indicated restriction enzymes and then ligated to a cassette including P<sub>ara</sub>, araC repressor, Cm<sup>R</sup> and the p15A replicon, which was amplified by PCR on pKJE7 (Takara) using as primers: 5′CTCGAGATATGCTAAACGTCTCCACAATCCATAGCCA (P<sub>ara</sub>-NdeI) and 5′GAGCCTCGGGTTACTGGACGGCGACAATCCG (P<sub>ara</sub>-XmaI).

Each figure panel is composed of: i) a linear scheme (not drawn to scale) outlining the plasmids (top); ii) quantitative Western-blotting assays using a monoclonal antibody against the N-terminal His6 tag common to the three proteins (bottom left); and iii) plots of the estimated average number of protein molecules per cell along the induction time course (right). Both expression vectors are based in compatible, low copy-number replicons (light grey: RK2-trfA in A and p15A in B), distinct antibiotic resistance markers (white: ampicillin-<sup>R</sup> in A and chloramphenicol-<sup>R</sup> in B) and independently inducible promoters (P<sub>tac</sub> in A and P<sub>ara</sub> in B), including the genes coding for their respective repressors (LacI<sup>q</sup> in A and AraC in B). For assessing the relative protein expression levels by quantitative Western blotting, OD<sub>600</sub> for bacterial cultures were measured at the indicated times when cells from 1 mL aliquots were harvested, resuspended in 125 µL of SDS-PAGE loading buffer and lysated by boiling during 10 min. Sample volumes equivalent to 0.01 OD<sub>600</sub> (≈ 2x10<sup>7</sup> cells), and 0.035 µg (0.55 pmol) of purified His6-Orc4p (Giraldo and Díaz-Orejas 2001) as standard for quantification, were then analyzed in SDS-PAGE gels (12.5% polyacrylamide). Western blotting was performed as described (Giraldo and Díaz-Orejas 2001), using a monoclonal anti-His tag antibody (Sigma; 1:30,000 dilution) and a secondary HRP-conjugated anti-mouse antibody (GE Healthcare; 1:10,000). After chemiluminescent reaction (ECL Plus kit, GE Healthcare), X-ray films (Agfa Curix RP2) were digitized and analysis was carried out with the Quantity One software package (v. 4.6.3; Bio-Rad). Data were plotted using KaleidaGraph (v. 3.6.2; Synergy Software).
Figure S7. Expression of the amyloidogenic RepA-WH1(A31V)-mRFP in the *E. coli* strain MC4100 or in isogenic mutants for the two chaperones under study: *dnaK756* or *ΔclpB*. Representative views from bulk culture aliquots taken after IPTG induction are displayed superposed to the DIC and red fluorescence images. Right column shows (red fluorescence emission) a 6-fold magnification of the sectors boxed in yellow. For details, see Fig. S6A.
Figure S8. Plots of quantifications performed on *E. coli* cells in microscope fields from three independent experiments carried out under the conditions assayed in Figs. S7 and S9. (A) The percentage of cells bearing red fluorescent foci. (B) The average cell length. (C) The proportion of cells bearing either globular or comet-like/irregularly shaped aggregates. (D) The distribution of the number of aggregates per cell. Counting was focused on samples collected at the time of protein induction (t=0) and 2 and 4 h afterwards. At least 100 cells were counted for each class and sample. Bracketed lines represent the standard deviations from the mean values, which are displayed as histogram bars.
Figure S9. Amyloidogenesis of RepA-WH1(A31V)-mRFP in the chaperone-deficient strains (Fig. S7) complemented by the bulk expression of either DnaK or ClpB from a compatible plasmid (Fig. S6B). IPTG and Ara inducers were added, in either order (+ind. 1 → +ind. 2), within a 30 min interval, which result in prior overexpression of the prionoid (Amyl → DnaK/ClpB) or a chaperone (DnaK/ClpB → Amyl), respectively. The Hsp70 chaperone partially disorganizes the globular prionoid particles into more diffused, comet-like aggregates (arrows).
Figure S10. Intracellular distribution of RepA-WH1-mRFP and the DnaK/ClpB chaperones explored by epifluorescence and confocal microscopies. (A) Complementation of dnaK756 and ΔclpB strains was performed with the DnaK and ClpB chaperones, respectively, by consecutive Ara → IPTG induction (Fig. S9). Endogenous mCherry (red) and extrinsic, immuno-labelled (green) fluorescence localize the prionoid and chaperone molecules, respectively. Nucleoid DNA staining with DAPI was also performed for confocal microscopy. (B) Color (RGB) channels, plotted along longitudinal sections (yellow dashed lines in A) through chaperone-expressing cells, highlight the mutually exclusive distribution of aggregates (red) and nucleoid (blue), and the differential location of DnaK and ClpB chaperones.

For immuno-fluorescent staining, cells were treated with lysozyme and incubated with antibodies as indicated (Fernández-Tresguerres et al., 2010), using as primary antibodies either a mouse monoclonal (mAb) anti DnaK (clone 8E2/2, Enzo Life Sciences; 1:1,000 dilution) or a rabbit polyclonal anti-ClpB (1:5,000) (Tek and Zolkiewski, 2002), and as secondary antibodies Alexa 488-coupled anti-mouse or anti-rabbit (Molecular Probes; 1:100). Incubation with the DNA-specific fluorophore DAPI (4′,6-diamidino-2-phenylindole; Serva) was carried out at 5 μg.mL⁻¹ for 20 min. Confocal images were acquired in a Leica TCS-SP2-AOBS microscope, mounting a HCX PL APO CS 100x (NA 1.40) objective. Epifluorescence microscopy was carried out as described in the main text, using the following additional filters and exposure times: Alexa 488 (EX 482/35, EM 536/40; 200 ms), DAPI (EX 387/11, EM 447/60; 2 s).
Figure S11. Distribution of His6-tagged RepA-WH1(A31V)-mRFP, DnaK and ClpB proteins between the aggregated (P) and soluble (S) fractions after sedimentation (16,000 g, for 30 min at 4 °C) of whole cells extracts from bacterial strains cultured as in Figs. S7 and S9. (A) Western-blot and (B) quantifications performed on two independent gels, both carried out as described in Fig. S6.

Movie S1. Propagation of the RepA-WH1(A31V)-mCherry prionoid in *E. coli* cells growing in microfluidic channels. Movie is composed of red fluorescence emission frames captured every 6 min along 72 h.

Movie S2. Aggregation as IBs of the amyloid-defective protein RepA-WH1(ΔN37)-mCherry in *E. coli* cells while growing in the microfluidic device. Movie was assembled from red fluorescence emission frames captured every 6 min along 10 h.

Movie S3. Propagation of the RepA-WH1(A31V)-mCherry prionoid in *E. coli* cells growing in the microfluidic channels in the presence of the IBs marker IbpA-YFP. Movie is composed of frames made by the superposition of the red (RepA-WH1) and yellow (IbpA, in false green to enhance contrast) fluorescence emission channels. Dual colour images were captured every 6 min along 10 h.

Movie S4. The effect of the DnaK inhibitor myricetin on the propagation of the RepA-WH1(A31V)-mCherry prionoid in *E. coli* followed through microfluidics. Movie is composed of red fluorescence emission frames captured every 6 min along 10 h.

Movie S5. Tracking by microfluidics the effect of DnaK chaperone overexpression on the propagation of the RepA-WH1(A31V)-mCherry prionoid in *E. coli*. Movie is composed of red fluorescence emission frames captured every 6 min along 14 h.

Movie S6. The effect of overexpressing the ClpB chaperone disaggregate on the propagation of the RepA-WH1(A31V)-mCherry prionoid in *E. coli* cells growing in microfluidic channels. Movie is composed of red fluorescence emission frames captured every 6 min along 18 h.
References


