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Addressing Intracellular Amyloidosis in Bacteria with RepA-WH1, a Prion-Like Protein

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Running Title: Bacterial Model of Amyloid Proteinopathy

Abstract

Bacteria are the simplest cellular model in which amyloidosis has been addressed. It is well documented that bacterial consortia (biofilms) assemble their extracellular matrix on an amyloid scaffold, yet very few intracellular amyloids are known in bacteria. Here we describe the methods we have resorted to characterize in *Escherichia coli* cells the amyloidogenesis, propagation and dynamics of the RepA-WH1 prionoid. This prion-like protein, a manifold domain from the plasmid replication protein RepA, itself capable of assembling a functional amyloid, causes when expressed in *E. coli* a synthetic amyloid proteinopathy, the first model for an amyloid disease with a purely bacterial origin. These protocols can be useful to study other intracellular amyloids in bacteria.

Key Words: Bacteria, Intracellular amyloid, Prionoid/Prion-like, Templated fibrillation, Dot/Western-blot, SDD-AGE, Time-lapse microscopy, Microfluidics, Immuno-electron microscopy

1. Introduction

When thinking about amyloids, especially on amyloid diseases, microorganisms do not come immediately to mind. However, yeast prions pop-up with contributions of paramount importance to our understanding of the molecular basis of the amyloid state and its epigenetic propagation. Regarding bacteria, the relevance of the functional extracellular amyloids in scaffolding biofilm formation, and thus in contributing to antibiotic resistance, is also insurmountable.

The folded N-terminal 'winged-helix' domain (WH1) of the plasmid DNA replication protein RepA [reviewed in **(1)**] has the property of assembling amyloid fibers *in vitro* upon allosteric binding to dsDNA effector sequences, while different mutations in the protein modulate its amyloidogenicity **(2-4)**. Based on these *in vitro* findings, here we dissect the *in vivo* methods that we have used along the last years to generate a fully bacterial model system for an intracellular amyloid disease, the RepA-WH1 prionoid (prion-like protein), and to analyze in *E. coli* cells the molecular determinants of amyloidogenesis, vertical propagation (mother cell-to-daughter cells) and toxicity [reviewed in **(5,6)**]. Its expression fused to a fluorescent protein resulted in the formation of cytotoxic protein aggregates that hampered bacterial proliferation **(7-9)**. We followed the growth of bacteria either in bulk liquid cultures, on solid agarose cushions **(7)** or in a microfluidic device ('mother cell machine') **(8)**. We characterized the amyloid character of the aggregates with conformation-specific antibodies **(10)**, the electrophoretic analysis under semi-denaturing conditions (SDD-AGE) **(8,11)** and the binding of an amyloidotropic fluorophore (BTA-1) **(8)**. By tuning the intracellular levels of the *E. coli* Hsp70 chaperone DnaK, we found that it participates in transforming the cytotoxic, highly amyloidogenic conformation of the prionoid into a less toxic one, compatible with the survival of bacteria during many generations **(8)**. Both types of RepA-WH1 aggregates behave as distinct amyloid strains, according to their morphology and affinity for BTA-1, and were clearly different to the standard bacterial inclusion bodies (IBs), because they do not co-localize with the IB tracer protein IbpA **(8)**. We have also studied the dominance in aggregation and in templating of different mutant variants of RepA-WH1 through co-localization experiments in vivo **(11)**. Apart from fluorescence microscopy, we have developed protocols to visualize by means of immuno-electron microscopy the intracellular location and co-localization with DnaK chaperone of the RepA-WH1 amyloid foci **(7,8)**. We found that RepA-WH1 amyloidogenesis initially occurs at the bacterial nucleoid **(10)**. Furthermore, we later discovered that, in the whole native RepA, the WH1 domain drives the assembly of an amyloid nucleoprotein complex that negatively regulates plasmid DNA replication, making such assemblies the first intracellular functional amyloid ever described in

bacteria, or related to DNA dynamics **(12)**. With the recent finding of yeast-alike prion domains in bacteria **(13,14)** the universe of intracellular bacterial amyloids has just started its expansion.

2. Materials

2.1. Expression of RepA-WH1 and chaperone co-factors

2.1.1. Plasmid constructs (**Note 1**).

1. pRG-*repA*-WH1(WT) and pRG-*repA*-WH1(A31V) (pMB9 replicon, high copy number, P_{tac} promoter with *lacI* operator, gene insertions between SacII and HindIII cloning sites, amplicillin selection) **(2,21)**.
2. pACYC-*repA*-WH1(WT) and pACYC-*repA*-WH1(A31V) vectors (p15A replicon, medium copy-number, insertion of the P_{tac} expression cassette from pRG vectors between SpeI and BamHI, $lacI^q$ repressor under its native promoter, chloramphenicol marker) **(11,18)**.
3. pSEVA121-*repA*-WH1(WT/A31V) vectors (RK2 replicon: *trfA* initiator protein gene plus *oriV* origin, low copy-number, SacII-HindIII expression cassette for the pRG vector series, $lacI^q$ repressor under its native promoter, ampicillin selection) **(17)**.
4. Monomeric fluorescent proteins mCherry **(15)** or YFP **(11)**, fused 3' to the *repA-WH1* constructs through a linker including a unique BspEI site.
5. Genes encoding the DnaK and ClpB chaperones amplified using as template genomic DNA from the *E. coli* K-12 strain MC4100, and primers having 5´-NdeI and 3´-XmaI sites using standard procedures **(20)**. Chaperone genes cloned into pACYC vector, including a P_{araBAD} promoter and its repressor (*araC*).
6. pSEVA511 vector for chromosomal insertions (R6K defective replicon: *ori*γ origin of replication, *Δpir*) **(17)**.

2.1.2. Bacterial strains

1. For expression from plasmids (batch cultures)
    1. *E. coli* K-12 strain MC4100 **(20)**: for co-expression of RepA-WH1 and chaperones.
    2. *E. coli* K-12 strain MDS42 **(21)**: the standard strain for synthetic biology of *E. coli* (**Note 2**).
2. For expression from the chromosome (microfluidic cultures)
    1. *E. coli* K-12 strain MG1655 **(19)**.
    2. *E. coli* K-12 strain DH5α-λ*pir* (*pir* replication initiator protein gene integrated in chromosome through a *lambda* prophage).

2.2. Bacterial cultures

2.2.1. Growth in batch

1. Luria Bertani (LB) rich medium **(20)**: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl.
2. Isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.2.2. Growth on agarose cushions

1. Luria Bertani (LB) rich medium, supplemented with bacto-agar to 1.5% **(20)**.

2.2.3. Growth in a microfluidic set-up

1. Polydimethylsiloxane (PDMS).
2. Trimethylchlorosilane (TMCS).
3. Oxygen plasma generator (as the 790 Series, Plasma-Therm, Inc., USA).
4. Minimal (M9) medium **(20)**: 1x M9 salts (128 g/l $Na_2HPO_4 \cdot 7H_2O$, 30 g/l KH_2PO_4, 5 g/l NaCl, 10 g/l NH_4Cl), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4% glucose. If required, supply with 10% (w/v) casamino acids (CAAs).

2.3. *In vitro* cross-seeding with *ex vivo* protein aggregates

2.3.1. Purification of intracellular RepA-WH1(A31V)-mCherry aggregates

1. Lysis buffer: 1.0 M NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% Brij-58, 1 mM pNH_2-benzamidine and 10% glycerol.
2. Wash buffer: 0.1 M NaCl, 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5% Na-deoxycholate, 0.5% N-dodecyl-N,N-dimethylammonio-3-propanesulphonate (SB-12), 1 mM DTT, 1 mM pNH_2-benzamidine and 10% glycerol.
3. Branson SFX250 sonifier.
4. Deoxyribonuclease I (DNase I).
5. Ribonuclease A (RNase A).
6. Aggregate storage buffer: 0.1 M Na_2SO_4, 4 mM $MgSO_4$, 20 mM Tris-HCl pH 8 and 10% glycerol.
7. Denaturing buffer: 6 M Gu·HCl and 20 mM Tris-HCl pH 8.

2.3.2. RepA-WH1 fiber assembly

1. Purified soluble RepA-WH1(WT) and RepA-WH1(A31V) **(2)**.
2. Fiber assembly buffer (2x): 0.1 M Na_2SO_4, 30 mM Tris-HCl pH 8, 4 mM $MgSO_4$, 7% polyethylene glycol (PEG)-4000 and 3% 2-methyl-2,4-pentanediol (MPD).

2.4. Antibody-based procedures

2.4.1. Dot-blot: non-denatured protein samples

1. Bio-Dot® 96-well microfiltration unit (Bio-Rad).
2. Nitrocellulose membrane, 0.45 µm diameter pore.
3. Whatman 3MM chromatography paper.
4. Bio-Dot microfiltration device.
5. Equilibration buffer: 0.1 mM Na_2SO_4, 40 mM HEPES pH 8.0, 5 mM $MgSO_4$.
6. TBST buffer: 20 mM Tris-HCl, 150 mM NaCl pH 7.5, 0.01% Tween-20 (w/v)
7. Blocking solution: 2% bovine serum albumin (BSA) in TBST.
8. Primary antibodies: rabbit polyclonal anti-WH1 and mouse monoclonal B3h7 **(10)**. Secondary antibody: HRP-conjugated goat anti-rabbit/mouse IgGs (GE Healthcare).
9. Enhanced chemiluminiscent substrate (ECL) Plus.

2.4.2. Western-blot: denatured protein samples

1. Gel electrophoresis mini-vertical device and parts.
2. SDS-PAGE sample buffer (5x): 0.25 M Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.05% bromophenol blue.
3. Polyvinylidene fluoride (PVDF) membrane.
4. Extra Thick Blot Filter Paper.
5. Transfer buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol.
6. Trans-Blot SD Semi-Dry Transfer Cell.
7. Primary antibodies: rabbit polyclonal anti-WH1 **(10)** and mouse monoclonal anti-His tag (Sigma-Aldrich). Secondary antibody: HRP-conjugated goat anti-rabbit/mouse IgGs.

2.4.3. Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE): conformational analysis of bacterial protein aggregates

1. Lysis buffer: 250 mM NaCl, 25 mM Tris-HCl pH 6.8, 5 mM EDTA, 10% glycerol, plus 1 tablet protease inhibitors per each 10 ml volume of buffer.
2. Loading buffer (4x): 0.5% TAE (40mM Tris, 20mM acetic acid, and 1mM EDTA), 5% glycerol, 2% sarkosyl, 0.1 mg/ml bromophenol blue, plus 1 tablet protease inhibitors.
3. Running buffer: 1x TAE, 0.1% SDS.
4. Trans-blot® Electrophoretic Wet Transfer Cell (Bio-Rad).

2.5. Visualizing prion-like aggregates in bacterial cells

2.5.1. Fluorescence microscopy of fixed bacterial cells

1. Bacterial cultures: *E. coli* K-12 strains MC4100 **(20)** and MDS42 **(21)**.

2. Phosphate-buffered saline (PBS, 1x): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4.
3. Fluorescence fixative: 2% formaldehyde (FA) freshly prepared from paraformaldehyde (PFA) powder in a fume cupboard. To dissolve FA, first warm the 1x PBS up to 60 ºC and then add the PFA powder. Once dissolved, filter the solution through a 0.20 µm filter and leave to cool down.
4. Immunofluorescence fixative: add Triton X-100 to the cold FA solution to a final concentration of 0.5%.
5. Permeabilization buffer: 1x PBS, 0.5% Triton X-100.
6. GTE buffer: 50 mM EDTA, 20 mM Tris-Cl pH 7.5, 50 mM glucose.
7. Lysozyme solution: Prepare 8 µg/ml lysozyme in GTE from frozen lysozyme immediately before use.
8. Blocking Buffer: 2% BSA in 1x PBS plus 0.05% Tween-20.
9. Amyloidotropic fluorescent dye: BTA-1 [2-(4´methylaminophenyl) benzothiazole], 1 mM stock solution in dimethyl sulfoxide (DMSO).
10. DAPI solution: 2-(4-amidinophenyl)-6-indolecarbamidine in 1x PBS containing 0.05% Tween-20.
11. Primary antibody solutions: primary antibody in blocking buffer (e.g. anti-His, 1:400; anti-WH1, 1:100; B3h7, 1:200; anti-DnaK, 1:1.000; anti-ClpB, 1:5.000).
12. Secondary antibody solution: Alexa 488-conjugated secondary antibodies in blocking buffer at 1:100 dilution.
13. Poly-lysine coated slides.
14. VECTASHIELD anti-fading mounting medium.
15. Transparent nail polish to seal the coverslips.
16. Fluorescence microscope Nikon Eclipse 90i equipped with an EFIPLAN APO VC 100x oil immersion objective and a Hamamatsu ORCA-R^2 CCD camera. Excitation and emission filters for the indicated fluorophores: m-Cherry (543/22, 593/40), BTA-1 (438/24, 483/37), DAPI (360/40, 470/40), and Alexa 488 conjugated secondary antibodies (480/40, 527/30). Differential interference contrast (DIC) setup.
17. Confocal Microscope: Leica TCS-SP2-AOBS with the Leica-confocal software.

2.5.2. Time-lapse microscopy of living bacteria

2.5.2.1. Agarose cushions set-up

1. Bacterial cultures: *E. coli* K-12 strains MC4100 **(20)** and MDS42 **(21)**.
2. Live cell image equipment: Leica AF6000LX consisting of a DM16000B inverted microscope with a HCX PLAN APO (100X/NA 1,40) oil immersion objective and a

Hamamatsu CCD camera, equipped with an incubation chamber with controlled humidity and temperature (37 ºC).

2.5.2.2. Microfluidic set-up

1. Bacterial culture: *E. coli* K-12 strain MG1655 **(19)**.
2. Inverted optics microscope (Nikon Eclipse Ti) within a temperature/humidity controlled box (Cube and Box, Life Imaging Services GmbH) equipped with a CCD camera (Photometrics Cool-Snap HQ2).
3. ImageJ software: https://imagej.net/

2.5.3. Immuno-electron microscopy of thin bacterial sections (iEM)

1. Bacterial cultures: *E. coli* K-12 strains MC4100 **(20)** and MDS42 **(21)**.
2. Agarose (low gelling temperature): 2% in 1x PBS.
3. Acrylic resin: LR White resin medium grade (London Resin Company).
4. Formvar-coated nickel grids.
5. Glycine buffer: 20 mM glycine in 1x PBS buffer pH 7.4.
6. Blocking Buffer: 2% BSA in 1x PBS buffer pH 7.4.
7. Primary antibodies solutions: in blocking buffer at adequate dilutions, usually 2 to 10 times more concentrated than for immunofluorescence (see 5.1.1.10).
8. Secondary antibody solution: 5 or 10 nm gold-conjugated secondary antibodies (Sigma-Aldrich) in blocking buffer, at 1:50 dilution.
9. EM staining solution: 5% aqueous uranyl acetate.
10. Ultramicrotome (Ultracut, Reichert).
11. Diamond knife (Diatome).
12. Electron microscope: JEOL 1230 EM.

3. Methods

3.1. Expression of RepA-WH1 and chaperone co-factors. To engineer a synthetic amyloid proteinopathy operating in *E. coli*, we took into account that A31V, a functional mutation enhancing DNA replication **(22)**, also boosted RepA-WH1 amyloidogenesis *in vitro* **(2)**. In addition, the amyloidogenicity of RepA-WH1(A31V) in bacteria could be enhanced by shifting its conformational equilibrium towards metastability by fusing to its C-terminus a domain other than WH2, naturally found in RepA **(7)**. In particular, the monomeric fluorescent protein mCherry **(15)** fulfilled that role and allowed for the intracellular location of RepA-WH1 aggregates by means of fluorescence microscopy **(7,8,11)**. A flexible linker, $(Gly\text{-}Ser\text{-}Ser)_2$-Gly, was included (in the dsDNA oligonucleotides encoding it, with a unique BspEI restriction site) to join the C-terminus

of RepA-WH1 and the N-terminus of mCherry, to allow for minimal interference between both in folding and/or aggregation. In such fusion protein, as characterized *in vitro* **(16)**, the mCherry tag does not directly contribute to aggregation: a fusion of mCherry to wild-type RepA-WH1 remained soluble and nontoxic *in vivo* **(7,11)**. Expression can be achieved either from plasmid vectors (see 2.1.1) or from genes inserted in the chromosome. In the case of insertion of the *repA-WH1* expressing cassettes in the chromosome, we proceeded as follow:

1. When a single copy gene was required to achieve maximal physiological control on expression, the expression modules (see 2.1.1) were inserted into the chromosome of the *E. coli* K.12 strain MG1655 **(19)**. The DNA fragments with those modules were digested with SpeI and EcoRI and cloned into pSEVA511 (Tc^R), a vector with the origin of replication of the plasmid R6K that is defective for the gene encoding its replication initiator protein (*pir*), and the same cloning sites of pSEVA121 **(17,23)**, and so as the pRK2 plasmid series. This plasmid was maintained in the *E. coli* strain DH5α-λ*pir*, which carries the *pir* gene integrated in the chromosome.
2. The pSEVA511-WH1(A31V)-mCherry derivative was then electroporated into the strain MG1655: Tc^R clones lacked any free plasmid, but this had been inserted into the chromosome. This was confirmed by PCR amplification using the same oligonucleotides used to clone the expression cassette. The resulting strains were transduced with supernatants of cell cultures that had been infected with the P1 bacteriophage, carrying a Cm^R marker and the YFP gene, to label the cytoplasm homogeneously with yellow fluorescence **(24)** (*see* **Note 3**).

3.2. Bacterial cultures

3.2.1. **Bacterial growth in batch**. Culturing bacteria in Erlenmeyer flasks is the default choice for any initial characterization of amyloidogenesis in bacteria.

1. Inoculate the strain of choice in 10 ml of Luria-Bertani (LB) medium **(20)**, supplemented with the selection marker at the appropriate concentration (e.g. ampicillin 100 μg/ml; kanamycin 50 μg/ml; chloramphenicol 30 μg/ml) (*see* **Note 4**).
2. Grow the culture o/n at 37 ºC with shaking. The next day measure the OD_{600} reached by the pre-inoculum and dilute it in fresh medium to OD_{600}= 0.05. Add the selection marker too. Grow bacteria at 37 ºC with shaking until the culture reaches exponential phase (OD_{600}= 0.2-0.3). For a standard K12 strain this usually takes 2h.
3. Induce your protein of interest with appropriate inducer according to your expression vector design. To induce RepA-WH1 expression from pRK2 and pACYC plasmids IPTG 0.5 mM was used when the culture reached OD_{600}= 0.2 **(11)**. For the successive

expression of chaperones and RepA-WH1, the promoter-specific inducers (0.1 m M IPTG and 0.5% l-arabinose) were added within a 30 min interval **(8)**.

4. Collect samples at different times post-induction (for RepA-WH1, each 30 min for 4 h) and process them accordingly to the technique of choice.

3.2.2. Bacterial growth on agar cushions. Growth on a thin agar cushion enables microscope monitoring of bacterial proliferation and protein amyloidogenesis, provided that the protein of interest is fused to a fluorescent tag.

1. Wash the slides and cover slips briefly in absolute ethanol, air dry and keep them at 37 ºC on a heating plate.
2. Add 75 µl of melted aerated 1.5% (LB) agar with 100 µg/ml ampicillin, and quickly drop a cover slip on top to obtain a thin cushion and let agar to solidify at RT (*see* **Note 5**).
3. Remove carefully the cover slip with tweezers.
4. Lay 10 µl of a freshly IPTG-induced batch culture on top of the cushion.
5. Cover with a clean cover slip and put inside the incubation chamber of the live cell image equipment. Specimens are kept in the microscope incubation chamber under controlled humidity and temperature (37 ºC). For visualization under the microscope, see 3.4.3.

3.2.3. Cell-to-cell propagation of RepA-WH1 within a microfluidic set-up. Microfluidics in a manufactured, comb-shaped chip multiplexed with microchannels, due to the laminar continuous flow of media (including nutrients, cofactors and drugs), enables the analysis of hundred of generations of bacteria while maintaining the single founder mother cell at the bottom of each channel. Thus, the cell-to-cell propagation of fluorescence-tagged protein aggregates, while maintaining the single founder mother cell at the bottom of the channel, can be visualized under an inverted microscope. This protocol is an expanded version of that described in **(8)**, but the interested readers should refer to specialized literature for further information **(25-27)**.

3.2.3.1. Chip fabrication

1. Soft lithography was used to fabricate the mold for the channels (1.1-1.3 µm width and 1.3-1.5 µm height), connected to a main channel of 25x100 µm (depth and width) and 30 mm (length). This structure was initially constructed on a silicon substrate at the Kavli Institute of Nanoscience, Delft University of Technology (The Netherlands). The mold was then replicated in polydimethylsiloxane (PDMS).
2. To transfer the patterns from the silicon chip to the PDMS surface, two PDMS replicas were necessary. A 10 µL volume of the hydrophobic chemical trimethylchlorosilane (TMCS) was dropped beside the silicon mold, sealed and hold for 10 min. After

hydrophobic treatment, 1:10 PDMS was poured on the chip and solidified by baking at 80°C for 2 h. The hydrophobic treatment greatly decreased the interaction of PDMS with silicon.

3. The structure obtained on the PDMS surface was the inverse of the silicon mold and was then used as the mold for the second PDMS replica. Then, a 2 min activation of the PDMS surface was conducted using oxygen plasma (70 W for 5 min), followed by hydrophobic TMCS treatment (*see* **Note 6**). After baking at 75°C for 2 h, the formation of a hydrophobic molecular film prevented adhesion between the two PDMS layers during peel off, and the second PDMS replication was easy to accomplish.
4. A cover slide and the plasma-activated PDMS device, both freshly activated with air plasma, were then bound each to the other. After over-night at 75°C, the bond between PDMS and glass was strong enough to support the pressure of the flow through the microchannels.
5. Before use, surface chambers were neutralized with PEG400 (20%), followed by minimal M9 medium (supplemented with 1.5% PEG400) to avoid adherence of the cells to the PDMS.

3.2.3.2. Bacterial proliferation within microchips

1. *E. coli* K12 MG1655 strain derivatives MG1655::A31V-YFP, MG1655::ΔN37-YFP, MG1655::A31V-*ibpA*, MG1655::A31V+p15A-*dnaK* or MG1655::A31V+p15A-*clpB* were grown overnight at 37°C in minimal medium supplemented with casamino acids (M9-CAA). Then 50 µl of each cell culture were diluted in 20 ml of fresh medium and grown to an OD_{600}**=** 0.2 (*see* **Note 7**).
2. A 1 ml volume of *E. coli* culture was concentrated by centrifuging at 2,000 rpm for 5 min and then washed twice with M9-CAA supplemented with PEG400 to 1.5% (*see* **Note 8**).
3. 100 µL of concentrated medium with a cell density of 10^{10} cells/ml was injected from one entrance of the channel. Check cell density in the main channel under a microscope to be sure it is crowded with bacteria. A high density of cells increases the number of channels that will host a mother cell in it after centrifugation.
4. By centrifugation at 2,000 rpm for 5 min, under a centrifugal force co-aligned with the chip surface (*see* **Note 9**), the *E. coli* cells were driven from the main channel into the micro-channels. After centrifugation, the PDMS film was immediately released from the metal plates (*see* **Note 10**) and then carefully reconnected to the main flow tubes (inlet/outlet). Usually, more than 80% of the channels contain a mother cell.
5. The ready-to-use microfluidic chip was casted in an inverted fluorescence microscope setting and bacterial proliferation was followed by time-lapse microscopy (see

3.5.2.2).

3.3. *In vitro* cross-seeding with *ex vivo* protein aggregates. This procedure tests the ability of aggregates generated *in vivo* to template fibril amyloidogenesis *in vitro* **(7)**. As conformational templating is a hallmark of amyloidogenesis, this assay probes the amyloid character of any given protein that aggregates intracellularly.

3.3.1 Purification of intracellular RepA-WH1(A31V)-mCherry aggregates

1. Inoculate two Erlenmeyer flasks (2 l nominal volume) containing 400 ml of LB medium plus ampicillin (100 µg/ml) with *E. coli* MG1655 cells carrying pRG-WH1(A31V)–mCherry, and grow the culture at 30 °C to OD_{600}**=** 0.8.
2. Add IPTG (0.5 mM), shift the temperature to 37 °C and grow over-night (OD_{600}**=** 4.0).
3. Harvest the cells by centrifugation (Sorvall SLA-1500, 5,000 rpm, 10 min, 4 ºC), and wash the pellet with cold 0.9% NaCl.
4. Resuspend the cell pellet in 15 ml of lysis buffer, add lysozyme (1 µg) and incubate the suspension at 37 °C for 30 min
5. Sonicate the lysate (two cycles, 30 s each, using a 1 cm wide tip) and clarify it by ultracentrifugation at 20,000 rpm in a Beckman Ti60 rotor, for 1 h at 4 °C.
6. Resuspend the pellet (i.e. the aggregated WH1(A31V)–mCherry protein) in 15 ml of wash buffer.
7. Sonicate the suspension and centrifuge as before.
8. Resuspend the pellet in 15 ml of the same detergent-free buffer supplemented with 5 units of DNaseI and 0.5 µg of RNaseA and then incubated for 1 h at 37 °C.
9. Sonicate the protein suspension and centrifuge again.
10. Resuspend the pellet in 10 ml of aggregate storage buffer and perform an additional sonication/centrifugation round.
11. Homogenate the aggregated protein in 1.5 ml of the detergent-free buffer.
12. Cast a sucrose discontinuous gradient: in a 2 ml Eppendorf tube, successively display 200 µl of 60 (bottom) > 50 > 40 > 30 > 20% (top) sucrose in the same buffer (see 12), freezing each layer at -70 ºC before adding the next one (*see* **Note 11**).
13. Carefully lay 100 µl of the suspension of aggregates on the top of the gradient, then leave to temper to 4ºC in the centrifuge rotor and spin the tubes at 7,500 rpm for 16 h at 4°C.
14. Carefully remove 100 µl aliquots from the top of the tube and analyze 10 µl by SDS-PAGE (*see* **Note 12**). RepA-WH1(A31V)–mCherry inclusions are found to peak at fraction 8 in the gradient.

15. Estimate protein concentration in the inclusions by dissolving 10 µl of the purified aggregates in denaturing buffer from the the absorption spectrum using the equation C (mg/ml) **=** 1.55 x A_{280} **-** 0.76 x A_{260} **(28)**. Purified protein inclusions with A_{280}/A_{260} ratio >1.8 are considered free of nucleic acids.

3.3.2. Seeding of RepA-WH1 fiber assembly

1. Display, in 500 µl Eppendorf tubes, 50 µl aliquots of a 25 µM preparation of soluble RepA-WH1(WT) or RepA-WH1(A31V) **(2)**. Dilute in the same volume of fiber assembly buffer (x2).
2. Supplement the protein samples with 1 µg of pure RepA-WH1(A31V)–mCherry inclusions.
3. Incubate samples at 4° C (in a fridge) and remove 10 µl aliquots at different time points (e.g.: 0, 1 h and 1, 7, 14, 21, 28 and 35 days).
4. Analyze assembly by electron microscopy with negative staining (see 3.5.3).

3.4. Antibody-based procedures. Antibodies, either specific of a particular protein antigen or a fused peptide tag, or recognizing a particular conformation in proteins [e.g.: amyloid-specific antibodies **(29,30)**] are valuable tools in the research of intracellular bacterial amyloids, both for assessing amyloidogenesis *in vitro* and bacterial cells *in situ* **(10)**.

3.4.1. Dot-blot: non-denatured protein samples

1. Set nitrocellulose membrane, previously hydrated with ultrapure milli-Q water, in a microfiltration device.
2. Pre-equilibrate the wells you will use with appropriate buffer, e.g. for RepA-WH1(A31V) samples, we used equilibration buffer (see 2.2.2), whereas wells loaded with denatured proteins were rinsed with the same buffer supplemented with 1% methanol (*see* **Note 13**). Apply vacuum.
3. Load protein samples diluted in equilibration buffer into the wells (0.2 µg, and subsequent 2-fold step serial dilutions of RepA-WH1(A31V), in 100 µl final volume) and spot them under gravity flow. Add, 0.1% of SDS to the dilution buffer for denatured control samples and boil for 4 min before blotting (*see* **Note 14**).
4. Include as controls insulin fibers 2 mg/ml (in formate buffer pH 1.2, heated at 60 °C for 2 h), microcine E492 fibers **(31)**, or purified RepA-WH1(A31V)-mCherry aggregates **(10)**.
5. Block the membrane at room temperature for 1 h, with blocking solution while still in the blot device and apply vacuum. If the membrane is removed prior to the washing step, incubate overnight at 4 °C (*see* **Note 15**). Continue in the step 5 if you perform

the protocol in the blot device; if you had removed the membrane, follow in the step 12 of the Western-blot protocol (see below).

6. Incubate 1h with the primary antibodies (0.1-10 µg/ml for purified antibody, 1:1000 to 1:3000 for hybridoma supernatants or antisera/ascitic fluid) in TBST plus BSA and apply vacuum (*see* **Note 16**).
7. Wash the membrane three times with TBST of 20 min each and apply vacuum.
8. Incubate with the appropriate HRP-conjugated secondary antibodies (for optimum dilution, follow the manufacturer´s recommendation) for 1 h and apply vacuum.
9. Wash the membrane three times with TBST of 20 minutes each and apply vacuum.
10. Disassemble the Bio-Dot device and follow from step 17 of the Western blot protocol (see below).

3.4.2. Western-blot: denatured protein samples

1. Grow bacterial cells as above (see 2.2.1).
2. Harvest cells by centrifugation (2 min at 13,500 rpm). Discard supernatant.
3. To 1 ml of 5x SDS-PAGE sample buffer **(32)**, add 50 µl of b-mercaptoethanol stock (14.3 M). Prepare 1 ml of 2x sample buffer by diluting the 5x stock with milli-Q water.
4. Resuspend the cell pellet in 2x sample buffer. For pellets from 0.2 ml of culture at OD_{600}= 0.3, resuspend cells in 100 µl final volume.
5. Lysate the cells by heating samples at 95 ºC for 10 min (*see* **Note 17**). Then cool on ice and spin down briefly.
6. Load 10 µl each sample onto a previously casted SDS-PAGE gel (10 cm x 8 cm plates size) **(32)**. The percentage of acrylamide/bisacrylamide depends on the size of your protein of interest (*see* **Note 18**). Run electrophoresis at 150 V for 60 to 90 min, depending of the size of our protein. Caution: Extreme care must be taken during electrophoresis to avoid any electrical hazard.
7. Cut a PVDF membrane adjusted to the size of the polyacrylamide gel and 2 pieces, with the same dimensions, of a thick filter paper.
8. Pre-wet the PVDF membrane with methanol for 30 s and immediately afterwards with transfer buffer. Leave it in water until the membrane goes to the bottom of the container (*see* **Note 19**).
9. Incubate the membrane and the gel in transfer buffer for 10-15 min at room temperature.
10. Assemble the transfer sandwich. Attach tightly the gel to the PDVF membrane and add on each side a piece of the blotting paper (*see* **Note 20**). Set it up directly on the surface of the semi-dry transfer-blot system. Pre-wet filter papers with transfer buffer and place over the anode (*see* **Note 21**).

11. Run the transference at 12 V for 2h. Use an electrophoresis power supply able to provide high current reads. Caution: Extreme care must be taken during electrophoresis to avoid any electrical hazard.
12. Dismantle the transference setting and incubate the membrane in blocking solution at 4ºC overnight.
13. Incubate the membrane with the primary antibody, diluted as convenient in fresh blocking solution, for 2 h at room temperature. The concentration depends on the affinity of each antibody and the amount of protein present in the sample. E.g., anti-His monoclonal antibody is used between 1/50,000-1/25,000 when H_6-RepA protein was expressed with 0.5 mM of IPTG.
14. Wash the membrane with TBST, 3 times for 20 min each.
15. Incubate the membrane for 1h at room temperature with a horseradish peroxidase-conjugated secondary antibody diluted in blocking solution, according to the specifications of the supplier.
16. Wash the membrane with TBST, 3 times 20 min each.
17. Incubate for 5 min with a luminiscence detection reagent. Remove the excess of liquid.
18. Place the membrane in a caster for X-ray films or in a luminiscence imager and expose, trying different exposure times according to luminiscence intensity.

3.4.3. Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE): conformational analysis of bacterial protein aggregates. SDD-AGE, originally developed at the laboratory of Susan Liebman to detect the presence of detergent (SDS)-resistant amyloid assemblies **(33)**, can be adapted to proteins carrying hydrophobic amyloidogenic stretches, such as the bacterial prion-like protein RepA-WH1 **(11,34,35)**.

1. Grow bacterial cells in batch (see 3.2.1).
2. Harvest bacterial cells from 25 ml of LB culture (OD_{600}= 2) by centrifugation and resuspend cells in 400 µl of lysis buffer.
3. Transfer the suspension to a tube containing silica (or glass) beads.
4. Lysate the cells using a mechanic homogenizer (4 cycles at maximum speed, 30 s each at 4 °C) (*see* **Note 22**). Dilute 3 µl of cell lysate with 30 µl of lysis buffer, and then add 10 µl of 4x loading buffer to the sample.
5. Incubate the samples 10 min at room temperature.
6. Prepare a 200 ml 1.5% agarose gel in 1x TAE buffer. Melt the suspension, cool to ≈ 65 ºC, add SDS to 0.1% and pour the slurry into a (20 x 24 cm) methacrylate tray mold. Make sure that the casting tray is on a flat surface (*see* **Note 23**). Once

jellified at room temperature, place into the electrophoresis cuvette filled with running buffer at 10 ºC.

7. Load the samples in the gel wells and run electrophoresis at 100 V for 7.5 h at 10 °C (*see* **Note 24**). Caution: Extreme care must be taken during electrophoresis to avoid any electrical hazard.
8. Cut a PVDF membrane adjusted to the size of the agarose gel and 2 pieces with the same dimensions of chromatographic paper.
9. Attach tightly the gel to the PDVF membrane and add on each side a piece of chromatographic paper. Every sheet must be pre-wet in transference buffer. Make sure that there are no bubbles trapped in between (*see* **Note 21**).
10. Place the whole cast into a wet transfer blot cell and cover with running buffer. Caution: Extreme care must be taken during electrophoresis to avoid any electrical hazard.
11. Run the transference at 16 V (400 mA) and 10 °C for 15 h.
12. Perform incubation with the appropriate antibodies to detect the protein polymers.

3.5. Visualizing prion-like aggregates in bacterial cells. The characterization of any propagating prion-like protein, either bacteria or eukaryotic cells, heavily relies on imaging techniques. In the case of the RepA-WH1 prionoid, we have resorted to optical (fluorescence) microscopy, taking advantage of mCherry tagged variants of the protein, and electron microscopy when higher resolution was demanded, although the latter implies working with fixed cells. Beyond the steps related to sample preparation, the following procedures are generic protocols amenable to different microscope settings and optical configurations.

3.5.1. Fluorescence microscopy of fixed bacterial cells. The protocols described in this section concern the identification of intracellular protein aggregates in bacteria, the survey of their potential amyloidogenic nature with an amyloidotropic fluorophore (BTA-1) **(7,8,11)**.

1. Sample 0.2 ml of a bacterial culture expressing RepA-WH1, measure OD_{600nm} and mix with the same volume of PBS. Centrifuge 10 min at 1,600 g.
2. Wash the pellet with 0.4 ml PBS and centrifuge 10 min at 1,600 g.
3. Add 0.2 ml of 2% FA in PBS (freshly prepared from PFA) to the pellet and fix for 30 min at room temperature (*see* **Note 25**).
4. Centrifuge at 1,600 g and wash the pellet twice in 0.2 ml PBS for 10 min.
5. For amyloid-specific BTA-1 staining, add to the washed pellet 0.2 ml of 0.5 mM BTA-1 in DMSO and incubate 30 min at room temperature in the darkness. Alternatively, add the BTA-1 solution for 20 min during fixation (*see* **Note 26**).

6. Lay 15 µl of serial dilutions (in PBS) of the fixed cell suspension onto 0.1% poly-L-Lysine coated slides and air dry.
7. For DAPI staining of DNA, add 20 µl of 1.5 µg/ml DAPI in PBS to the slide, incubate for 15 min at room temperature and wash three times with 20 µl PBS. Then air dry (*see* **Note 26**).
8. Mount with VECTASHIELD and store slides at 4 ºC in an opaque box to avoid the loss of fluorescent signal.
9. Examine the samples in a confocal or epifluorescence microscope equipped with appropriate filters. Capture DIC images from the corresponding fluorescence fields.

3.5.2. Immunofluorescence microscopy of fixed bacteria. The procedures reported in this section allow for the labeling of subcellular structures with antibodies and the low-resolution location of specific prionoid-associated proteins **(7,8)**. All steps are performed at room temperature, except noted otherwise. Negative controls are performed by omitting the primary antibody, or by using bacterial cultures not expressing the protein.

1. Mix 0.2 ml of a bacterial batch culture (see 3.2.1) with the same volume of PBS and centrifuge 10 min at 1,600 g.
2. Wash the pellet with 0.4 ml PBS and centrifuge 10 min at 1,600 g.
3. Add 0.2 ml of 2% FA in PBS containing 0.5% Triton-X100 to the pellet and fix for 30 min at room temperature.
4. Centrifuge at 1,600 g, wash the pellet three times for 10 min in 0.2 ml PBS + 0.5% Triton-X100 and resuspend the pellet in GTE buffer.
5. Lay 15 µl of the culture onto 0.1% poly-L-Lysine coated slides and air dry.
6. In a humid chamber add 20 µl of freshly prepared 8 µg/ml lysozyme in GTE buffer and, after 5 min, remove the lysozyme solution and quickly wash twice with PBS.
7. Block with 20 µl of blocking buffer for 30 min at room temperature.
8. Remove the blocking solution and add 20 µl of the primary antibody at the appropriate dilution in blocking buffer. Incubate for 3 h at room temperature, or overnight at 4 ºC, depending on the antibody. For multiple labeling experiments incubate the antibodies, generated in different species, together.
9. Remove the primary antibody solution and wash with 20 µl PBS containing 0.05% Tween-20 for 5 min, ten times.
10. Incubate with 20 µl of the corresponding Alexa-coupled secondary antibody solution in blocking buffer at 1:100 dilution for 1 h at room temperature in the dark. For multiple labeling experiments, add the specific secondary antibodies with different fluorochromes together (*see* **Note 27**). From this step onwards, the slides should be protected from light.

11. Wash with 20 µl of PBS containing 0.05% Tween-20 for 5 min, ten times.
12. If DNA counterstaining is required, add 20 µl of 1.5 µg/ml DAPI in PBS containing 0.05% Tween-20.
13. Wash with 20 µl PBS for 10 min, three times.
14. Air dry and mount as above (see 5.2.1.8).
15. Examine the samples as above (5.2.1.9).

3.5.3. Time-lapse microscopy of living bacteria. Time-lapse microscopy is an essential tool to survey the vertical (mother-to-daughter cells) transmission and the intracellular dynamics of prion-like proteins in bacteria. We present here two alternative yet complementary assays to visualize intracellular bacterial aggregates *in vivo* with low (agarose cushions) and high (microfluidics) throughput. Readers interested in the subsequent image analysis must refer to specific literature on how to identify individual cells in each image (segmentation) and on procedures to track along time specific cellular features **(36,37)**.

3.5.3.1. Agarose cushions set-up

1. Prepare a thin cushion of 1.5% LB-agar with 100 µg/ml ampicillin as indicated (see 3.2.2.2). Then put it inside the incubation chamber of the live cell imaging equipment. Specimens are kept in the microscope incubation chamber under controlled humidity and temperature (37 ºC).
2. Select a group of cells in the preparation and program the microscope to capture images automatically at different intervals (10-15 min) for a maximum of 4 h after induction with IPTG. Use the excitation and suppression filters (BP546/2, BP600/40) for mCherry fluorescence. Take also phase contrast/differential interference contrast (DIC) images of the same field at the distinct intervals.
3. Use the software of the microscope for the superposition of TIFF images of the distinct channels. ImageJ **(38)** was used to edit the images as videos.

3.5.3.2. Microfluidic set-up

1. Once loaded with bacterial cells (see 3.2.3.2), place immediately the microchip under a temperature controlled (37 ºC) automated inverted microscope.
2. At 100x magnification, focus on multiple (e.g., 15) fields located at the microfluidic channels and initially containing a single cell. Fix the acquisition parameters (including exposure times and filter settings).
3. Circulate a continuous (2 ml/h) laminar flow of filtered M9-CAA at 37 °C through the setting by means of syringes coupled to inlet valves. When required, inject antibiotics or other molecules of interest.

4. Using any automated time-lapse acquisition software, follow bacterial growth at the microchannels for 10-72 hours. Take at fixed intervals (1-10 min) image frames across the chip.
5. Correlative TIFF images were edited as movies using ImageJ **(38)**.

3.5.4. Immuno-electron microscopy of thin bacterial sections (iEM). The unmatched resolution achieved by TEM is invaluable to explore the fine structure of intracellular prion-like aggregates, in particular when cells have the reduced dimensions of bacteria (*E. coli* has an sphero-cylindrical shape with ≈ 1 µm section). In addition, the combined use of specific primary antibodies with secondary antibodies conjugated to gold nano-particles allows placing specific proteins in thin ultra-microtomic sections of the bacterial cells **(7,10-12)**. All steps are performed at room temperature if not stated otherwise. Negative controls are performed by omitting the primary antibody, or by using bacterial cultures not expressing the protein.

1. Wash the bacterial culture in PBS as described (see 3.5.2.2).
2. Fix the bacterial pellet in 2% FA in PBS for 1 h (see 3.5.2.3).
3. Centrifuge and wash the pellet three times (see 3.5.2.4).
4. Embed the fixed pellet in 2% low-melting point agarose. Heat the agarose in milli-Q water until it dissolves, then cold down to 37 ºC, add 10 µl to the centrifuge tube containing the pellet and resuspend the fixed bacteria.
5. Once the agarose jellifies on ice, carefully take out the gel, cut it into pieces and transfer to a 2 ml centrifuge tube.
6. Wash the gel pieces with 1 ml PBS for 30 min at room temperature.
7. Dehydrate the samples at 4 ºC by passing them through an ethanol series: 30% ethanol for 30 min; 50% ethanol for 30 min; 70% ethanol for 30 min to overnight; 90% ethanol for 45 min; three times 100% ethanol for 45 min.
8. Infiltrate in LR White resin in three steps at 4 ºC (*see* **Note 28**):
    a. 100% ethanol: LR White resin 2:1 for 2 h.
    b. 100% ethanol: LR White resin 1:2 for 2 h.
    c. LR White resin for 3 days, changing the resin every 12 h.
9. Close the samples in gelatin capsules with LR White resin eliminating the air and cure them at 60 ºC for 20-22 h.
10. Cut ultrathin sections on an ultra-microtome with a diamond knife and mount them on nickel grids coated with a Formvar film (*see* **Note 29**).
11. For immuno-gold labeling, float the grids successively on 10 µl drops of the adequate solutions on Parafilm, with the sections facing the liquid in a humidity chamber, as follows:
    a. Incubate on PBS containing 20 mM glycine, to quench aldehyde groups.

b. Wash on PBS containing 0.05% Tween-20 for 10 min.

c. Block on blocking buffer (PBS containing 0.05% Tween 20 and 2% BSA) for 30 min at room temperature.

d. Incubate the grids on drops of the primary antibody at the appropriate dilution on blocking buffer for 1 to 3 h at room temperature. For multiple labeling, add the antibodies produced in different species together (*see* **Note 27**).

e. Wash 3 times for 10 min on PBS with 0.05% Tween-20.

f. Incubate with the secondary gold-coupled secondary antibody at the appropriate dilution in blocking buffer for 1 h at room temperature. In the case of multiple labeling, use different gold particle sizes for the different secondary antibodies (note 5).

g. Wash 3 times for 10 min on PBS with 0.05% Tween-20.

h. Wash the grids 3 times for 10 min on milli-Q water and dry them for 10 min on a filter paper.

i. Contrast the samples for 30 min in 5% aqueous uranyl acetate in the dark. The solution should be centrifuged before use at 6,000 g for 30 min, to avoid any contamination particles in the preparation. Wash the grids 3 times for 10 min in milli-Q water and let them to dry.

12. Examine the grids in a transmission electron microscope operating at 80 kV.

4. Notes

1. Look for (or generate) the same unique restriction sites for the whole set plasmid vectors you plan to use. Although it might seem worthless at the beginning, this strategy allows you to create interchangeable modules between different plasmid expression vectors. This could be really useful to test differences in protein expression levels or to change in a single step only the relevant parts of your expression cassette, such as the reporter gene. This sort of standardization effort is at the core of Synthetic Biology, such as in the Standard European Vector Architecture (SEVA) initiative **(17,23)** (http://wwwuser.cnb.csic.es/~stflow-project/ST-Flow/Resources.html). In the case of the studies performed with RepA-WH1, modularity allowed us to easily exchange the expression cassette from pRK2 (pSEVA) to pACYC plasmids, using PCR and subsequent SpeI and BamHI digestion, as well as to change the fluorescent reporter from mCherry to YFP in the pRG plasmids, using a unique BspEI site between RepA-WH1 and the reporter gene. A general workflow was to initially clone the constructs with the prion-like protein into the pRG vectors, to then transfer their whole expression cassettes to pRK2 and finally, if required, to pACYC.

2. In the first report on the toxicity of the RepA-WH1 prionoid in a 'wild-type' *E. coli* K-12 strain (MC4100), we noticed that an insertion sequence had jumped from the bacterial chromosome into the *repA-WH1* ORF, as an efficient (10% of the clones upon the first transformation) and quick way for bacteria to get rid of the cytotoxic prionoid **(7)**. Since then, we use preferentially the *E. coli* K-12 strain MDS42 because, as it was edited to remove all the mobile genetic sequences from its parental strain **(21)**, stable expression of RepA-WH1 is achieved.
3. It is convenient to check the expression of a few independent clones: since the place of insertion in the chromosome using the aforementioned procedure is random, the strength of expression (protein levels achieved, addressed as fluorescence signal under the microscope and by Western blotting) is potentially affected by the genomic context and can sensibly vary.
4. Independent experiments carried out in minimal (M9) medium **(22)** achieved the same results.
5. Using agarose (electrophoretic quality) instead of agar works equally well and can be an issue if you observe excessive auto-fluorescence at a particular wavelength. These experiments can also be carried out in M9 medium (*see* **Note 4**).
6. In this process, each hydroxyl group on the silicon surface, formed as a monolayer after plasma bombardment, reacted with a silane group of TMCS.
7. To minimize the lost of founder cells from the channels, it is convenient to use a *ΔfliC* mutant, which lacks flagella and thus exhibits reduced mobility.
8. Liquid flow must be laminar, without resistance. Carefully to wash out all possible particles remaining inside the PDMS chip. An increase in pressure in this step could cause your chip to leak and therefore made it useless.
9. Be sure that the part that is parallel to the centrifugal force is the main channel of the chip. Take into account that, once you prepare the chip, the coverslip edge and the main channel of the chip may not be parallel.
10. The easiest way to carry out this step is by tapping the coverslip to the metal part of the centrifuge. Then, carefully remove the chip by cutting the tape using a razor blade.
11. The sucrose gradient is pretty stable while stored at -70 ºC, but once thawed from to 4 ºC samples must layered and centrifuged straightaway.
12. It is very important that, after removing carefully the tubes from the centrifuge to a vertical stand (grid), to pipet slowly just from the very surface (meniscus) of the solution. Do not try to punch the bottom of the Eppendorf tubes to remove fractions, because this will severely distort the gradient. If a swinging bucked rotor is not available, pelleted aggregates can be found attached to the tube wall opposite to the

centrifuge force. In such a case, scrap the aggregates with a yellow micropipette tip, once the liquid was removed.

13. The 96-well Bio-Dot® Bio-Dot SF microfiltration unit provides easy, reproducible protocols for binding proteins or nucleic acids in solution onto membranes. The Bio-Dot SF apparatus focuses sample to a thin line instead of a circle, making quantitation by densitometry more reproducible.
14. Include methanol in the buffer to allow the denatured proteins to attach to the membrane efficiently.
15. To perform the assay in the 96-well Bio-Dot® Bio-Dot SF microfiltration device is recommended to use no more than 2% BSA because otherwise the pores of the membrane could collapse. This problem is exacerbated if skimmed milk is used instead of BSA.
16. You should choose a specific antibody against your protein of interest. A recommended strategy, if the user is going to generate a recombinant protein, is to include a tag in the amino or carboxyl terminal of the protein and use a specific antibody against the tag. One example is the Histidines-tag, the corresponding antibody would be a Monoclonal anti-poly-histidine (E.g., Sigma-Aldrich, catalog number: H1029).
17. Samples could be stored frozen at -80ºC before lysis, or -20ºC once you boiled them.
18. For big size proteins cast poly-acrylamide gels between 8-6%. For small proteins (15-20 kDa) do it at 15%.
19. Avoid any dry area remaining in the membrane while rising in methanol by sliding the membrane slowly into the solution starting from one of its edges.
20. SDS provides a (roughly) uniform negative charge to proteins, proportional to their sizes. Thus, be sure that the membrane is on the positive side in the transfer blot system.
21. Make sure that there are no air bubbles trapped in the sandwich by eliminating the bubbles with a Pasteur pipette acting as a glass roller. Otherwise, presence of air bubbles will result in "ghost" areas in the detection film/image at the end of the protocol.
22. To ensure lysis of all bacterial cells present in the sample, a lysozyme treatment can be done previously to the mechanic lysis. Add 20 µl of 10 mg/ml lysozyme to each 400 µl of the lysis suspension and incubate for 10 min at room temperature. Keep samples on ice until the mechanic lysis step is performed.
23. Heat the agarose suspension to the point of melting and then add SDS to 0.1%. After pouring the agarose, eliminate all the possible air bubbles with a pipette tip.

24. For better detection of oligomers established through hydrophobic interactions, run electrophoresis at a lower voltage (50 V) and at room temperature for 12 h with 1% Sarkosyl in the sample (4% Sarkosyl in the loading buffer). The running buffer is the same described in the protocol.
25. FA is the best choice fixative for immunolabeling. It is a mono-aldehyde that penetrates the cells fast and its cross-linking ability is lower than that of glutaraldehyde, leaving more antigen sites that are recognized by antibodies.
26. After BTA-1 and DAPI staining, keep the slides protected from light.
27. For double labeling, it is important to choose the antibodies wisely. The primary antibodies should be produced in different species and the fluorochromes, or sizes of gold particles, conjugated to the secondary antibodies should be different for each protein to be detected. For iEM microscopy, the most commonly used secondary antibodies are conjugated to 5 and 10 nm gold nano-particles, albeit larger ones are also available.
28. Polymerized acrylic resins such as LR White are optimal for immuno-gold labeling of sections because they are hydrophilic and facilitate penetration of the antibody solution and recognition of epitopes by antibodies on the surface of the section.
29. Nickel grids are preferred to copper grids for post-embedding immunolabeling, since copper ions may have an inhibitory effect on antibody binding to the antigen.

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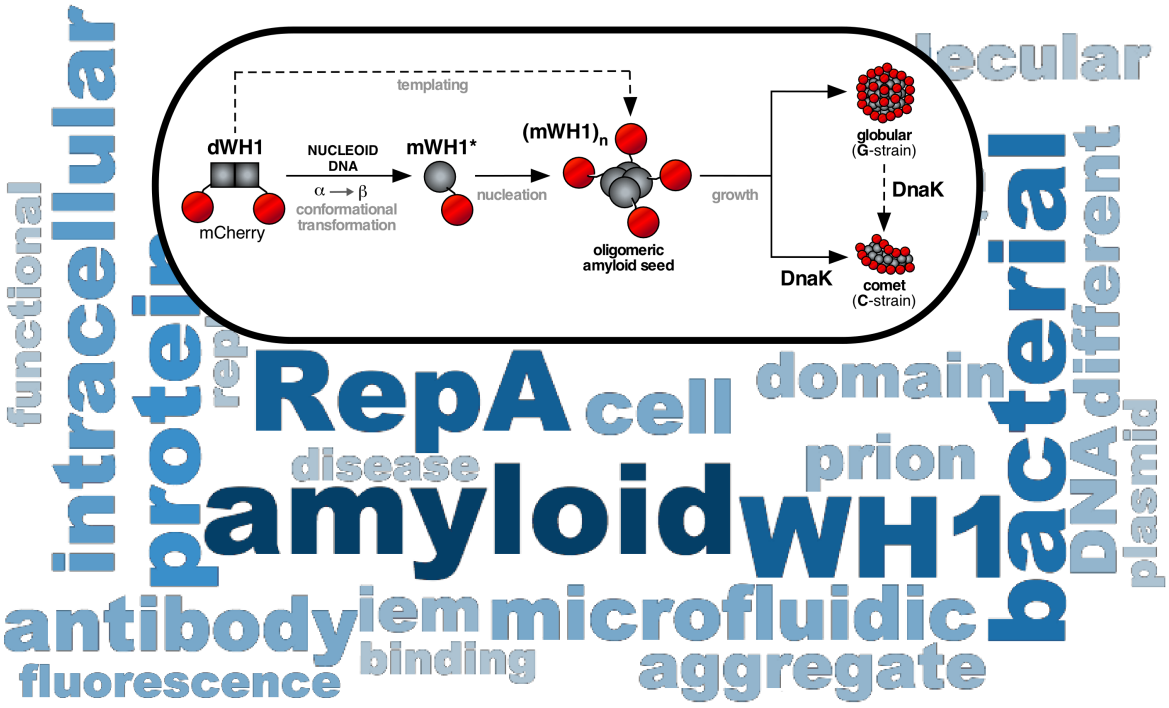

Figure 1. Schematic representation of an *E. coli* cell showing the major events in the amyloidogenesis of the prion-like protein RepA-WH1. Background: a word cloud plot (WordSift.org) of the main terms used in chapter.