

Composition and liquid-to-solid maturation of protein aggregates contribute to bacterial dormancy development and recovery

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Recalcitrant bacterial infections can be caused by various types of dormant bacteria, including persisters and viable but nonculturable (VBNC) cells. Despite their clinical importance, we know fairly little about bacterial dormancy development and recovery. Previously, we established a correlation between protein aggregation and dormancy in *Escherichia coli*. Here, we present further support for a direct relationship between both. Our experiments demonstrate that aggregates progressively sequester proteins involved in energy production, thereby likely causing ATP depletion and dormancy. Furthermore, we demonstrate that structural features of protein aggregates determine the cell's ability to exit dormancy and resume growth. Proteins were shown to first assemble in liquid-like condensates that solidify over time. This liquid-to-solid phase transition impedes aggregate dissolution, thereby preventing growth resumption. Our data support a model in which aggregate structure, rather than cellular activity, marks the transition from the persister to the VBNC state.

Bacteria have evolved diverse strategies to survive antibiotic treatment. The most well-known strategy is antibiotic resistance in which genetic changes allow cells to grow in the presence of antibiotics¹. Besides resistance, bacteria can endure antibiotic treatment by adopting a tolerant persister state. Persister cells are phenotypic variants in an isogenic population that survive lethal antibiotic exposure. They are characterized by no or slow growth and typically constitute a small fraction of the population. Moreover, persisters can revert to the antibiotic-susceptible, growing state, which allows them to recolonize the environment when the antibiotics are removed². This temporal tolerance renders persister cells important contributors to treatment failure and infection relapse^{3–7}. In addition to several active mechanisms that allow persisters to survive

antibiotic treatment, such as efflux pumps^{8–13}, persister cells are generally assumed to be tolerant because they reside in a dormant state^{14–16}. Indeed, persister cells exhibit low metabolism^{8,17}, reduced energy levels^{18–20} and no or slow growth^{8,21}. Emerging evidence suggests that persistence is not the end-stage of dormancy. Rather, it is hypothesized that persisters correspond to a shallowly dormant state and can further transition into a deeper dormant viable but nonculturable (VBNC) state from which they cannot easily recover to resume growth^{22–26}. VBNC cells are a subpopulation of nongrowing cells that show metabolic activity, but that cannot restart growth on standard laboratory media that would otherwise support their proliferation. Instead, they need a specific trigger to switch back to the antibiotic-susceptible, growing state^{27,28}. Because of their antibiotic

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tolerance and regrowth potential, VBNC cells have also been associated with the chronic nature of infections^{29,30}.

Because both persister and VBNC cells have been associated with the recurrence of infections^{3–6,29,30}, it is important to understand how the transition to these different dormant states occurs. It was demonstrated that dormancy is correlated with protein aggregation^{19,24,25,31–38} and the hypothesis was put forward that aggregation could drive dormancy development^{24,25}. Aggregation is suggested to do so by sequestering, and thereby depleting, proteins involved in key cellular processes, leading to the shutdown of vital pathways and the induction of dormancy^{24,25,35,39}. This hypothesis is supported by the observation that aggregates contain various proteins involved in essential metabolic processes, energy metabolism, transcription and translation^{19,24,25,33,37}. Nevertheless, no clear link between the aggregation of such proteins and the shutdown of their related cellular pathways has been demonstrated. Besides the limited understanding of the mechanism by which aggregation potentially induces dormancy, it also remains unclear how dormant cells that contain protein aggregates recover and regrow. Previous studies indicate that persister cells remove their aggregates before resuming growth^{24,25,37,38} and that the chaperones DnaK and ClpB are involved^{24,38}. However, it remains unknown if other chaperones and proteases can stimulate disaggregation and if other factors such as aggregate structure also influence the disaggregation efficiency. While aggregate structure and its relation to functionality has been thoroughly investigated in eukaryotes^{40–42}, the structure of aggregates and how it relates to dormancy has not been extensively investigated in bacteria.

Here, we present data that provide support for a role of aggregation in the induction of dormancy in *E. coli*. First, by perturbing central regulatory and metabolic pathways, we show that the relative timing of aggregation and dormancy is strongly conserved in strains that display different dynamics of dormancy development. Second, we confirm that aggregates are selectively enriched in proteins active in energy production and we demonstrate that the recruitment of these proteins in the aggregates precedes cellular energy depletion and persistence. Third, directly interfering with protein aggregation by manipulating the cellular level of the chaperone DnaK also accelerates or delays dormancy. Fourth, we confirm that aggregates are removed before cells can resume growth. Finally, we show that the ease with which these aggregates are removed—and thus also the regrowth potential of dormant cells—is determined by the structure of the aggregates. We show that, as starvation continues, proteins first assemble into liquid-like condensates that further solidify into aggregates. This solidification impedes disaggregation and thereby restricts regrowth of dormant cells over time. Our results strongly suggest that this aggregate solidification and the corresponding reduced recovery underly the transition from the persister to the VBNC state. This indicates that, contrary to the current hypotheses, VBNC cells are not characterized by lower energy levels compared to persister cells, but rather by aggregate solidification and a decreased recovery potential.

Results

Relative timing of protein aggregation and dormancy development are conserved in different genetic backgrounds

Dormant bacteria often contain protein aggregates^{19,24,34,37,38}. It was demonstrated by multiple studies that dormancy is correlated with protein aggregation^{19,24,25,31–38}. Moreover, it has been suggested that protein aggregation might be the driving force behind dormancy development^{24,25,39}. However, conclusive proof of the causal relationship between aggregation and dormancy is still missing. If there is a causal relationship between both, we expect that aggregation precedes dormancy and that the sequence of these events is conserved in strains with different dormancy dynamics. To test this hypothesis, we compared the timing of dormancy and aggregation in various genotypic backgrounds of *E. coli*. We chose to delete or overexpress genes

that not only alter persister levels, but also have different roles in central regulatory and metabolic processes. These genes include *rpoS*^{43–45}, encoding a general stress response regulator; *sdhC*⁴⁶, involved in energy production through both the TCA cycle and the electron transport chain; *recA*^{13,43,47,48}, important for DNA repair, and *obgE*^{25,49}, encoding a GTPase involved in ribosome assembly and cell cycle regulation. As a control, we also included a strain overexpressing the Obg-related GTPase Era⁴⁹ with no effect on persistence. We quantified the number of persister and VBNC cells as described before²⁵ (Fig. 1a, b, Supplementary Figs. 1 and 2). These results demonstrate that different genotypes display different dynamics of persister development. While almost all strains exhibit a similar increase in persistence during the transition from exponential to stationary phase, the timing at which they reach their peak persister level in stationary phase strongly varies. Despite these differences in the timing of reaching peak persister levels, most strains share a comparable maximal persister level. Consequently, the strains, previously identified as low or high persister strains, are therefore better defined as slow or fast persister strains, respectively.

Next, we wanted to verify whether different dynamics of persistence correspond to a different timing of aggregate development. Aggregates can be identified at an early stage using a fluorescent fusion of the small chaperone IbpA to msfGFP⁵⁰ (IbpA-msfGFP, referred to as IbpA aggregates), while late-stage aggregates can be observed as phase-bright foci (referred to as Ph aggregates)²⁵. The early detection of IbpA aggregates is due to the rapid association of IbpA with the aggregates⁵¹ and the higher sensitivity of fluorescence microscopy compared to phase contrast microscopy. We measured the percentage of bacterial cells carrying at least one aggregate for the selected strains using these two different readouts (Fig. 1c–f). To have a more succinct overview of the relative timing of aggregate formation and dormancy, we summarized our data using key timepoints for all processes under investigation (i.e. start and maximal level). Nonlinear logistic growth models were fitted to the data for IbpA and Ph aggregation to determine when aggregation started and when the maximum level was reached (Fig. 1c–f and Supplementary Table 1). We similarly plotted the timing of VBNC cell formation as the period between the start (indicated by the first drop in CFU levels) and the maximal level (indicated by the time when a stable number of CFUs was reached under our conditions) (Fig. 1g). This analysis confirms that both aggregation and dormancy happen progressively²⁵; protein aggregates are first marked by IbpA in an early stage of development and later mature into Ph aggregates, while persistence is followed by the onset of VBNC cell formation. Moreover, our results also demonstrate that the start of aggregation precedes or coincides with the peak in persistence in all tested strains, suggesting that aggregation might induce persistence. Strains with a deletion of *rpoS*, *sdhC* and *recA* and the strain with overexpression of *obgE* affect the timing of both aggregation and dormancy similarly (Fig. 1g). Specifically, the deletion of *rpoS* and overexpression of *obgE* accelerate both dormancy and aggregation, while the deletions of *sdhC* and *recA* delay both processes. Importantly, our results demonstrate that the increase in persistence in stationary phase is preceded by, and might therefore be linked to, protein aggregation.

To further verify if the timing of dormancy and aggregation are linked, we calculated the correlation between the time points of maximal persistence, IbpA aggregation and Ph aggregation and the starting point of VBNC cell formation in the different strains (Fig. 1h). This correlation analysis showed that the timing of aggregation and dormancy is significantly and highly correlated. These results support our previous findings that protein aggregation might induce dormancy. It can be noted that the timing of aggregation and persistence is less comparable in the $\Delta rpoS$ strain, suggesting that aggregation alone does not necessarily dictate the timing of persistence. Instead, it may involve reaching a specific threshold, potentially defined by the

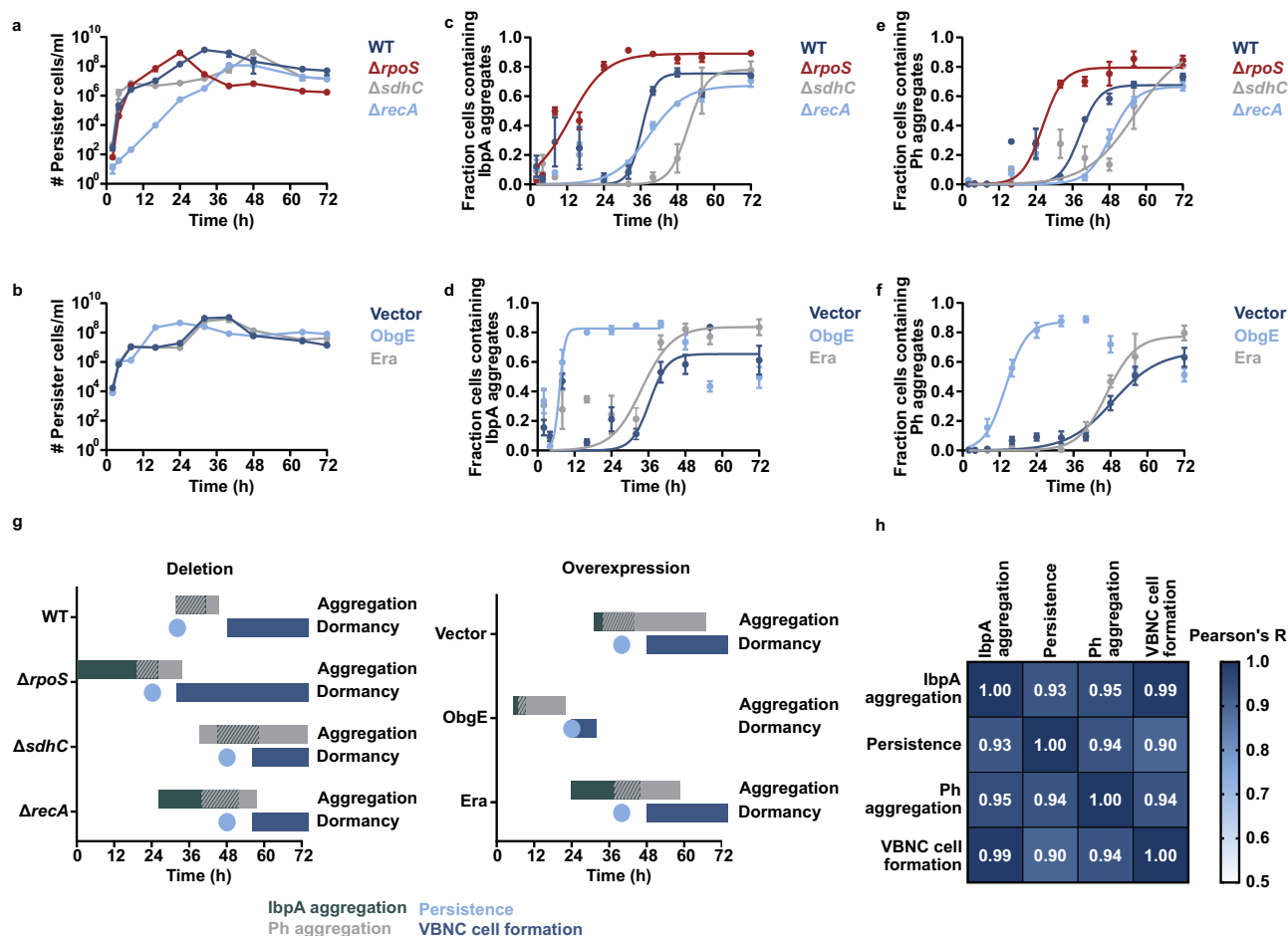


Fig. 1 | Dormancy and aggregation are dynamic processes that are often highly correlated. The strains analyzed for their effect on dormancy are the wild-type *E. coli* strain (WT), *E. coli* $\Delta rpoS$, $\Delta sdhC$ and $\Delta recA$ and *E. coli* with pBAD33Gm (Vector), pBAD33Gm-*obgE* (ObgE) and pBAD33Gm-*era* (Era). **a, b** The absolute number of persister cells that could grow after ofloxacin treatment was measured at different time points during incubation. Data are represented as averages $\pm$ SEM ($n \geq 4$) (See Supplementary Table 2 for exact values). Relative values of persisters, VBNC and dead cells are shown in Supplementary Fig. 1. **c–f** Quantitative microscopy analysis was used to determine the fraction of cells with IbpA (**c, d**) and Ph (**e, f**) aggregates at different time points during incubation. A minimum of 50 cells was analyzed for every repeat and every time point. A nonlinear logistic growth model was fitted on the data (solid line). Data are represented as averages $\pm$ SEM, ($n \geq 3$) (See

Supplementary Table 3 and 4 for exact values). **g** For each strain, the moment of maximal persistence and the start and maximal levels of VBNC cell formation and IbpA and Ph aggregation were determined and visualized. The moment of the start and maximum of IbpA and Ph aggregation were determined by fitting a nonlinear logistic growth model on the aggregation data and calculating the time between reaching 10% and 90% of the maximal value. **h** Heatmap of the Pearson's R values that were calculated based on the timing of maximal persistence, the onset of VBNC cell formation and the peak in IbpA and Ph aggregation across the selected strains ($n = 7$). All correlations were high and significant ($P < 0.05$) (See Supplementary Table 5 for confidence intervals and p-values). Source data are provided as a Source Data file.

sequestration of proteins involved in energy production, to induce persistence

After establishing that changes in dormancy are associated to similar changes in aggregation, we checked if overexpression of *era*, which was shown to not affect persistence⁴⁹, had an effect on the aggregation level in the cells. We observed that overexpression of *era* indeed did not affect the timing of dormancy. However, to our surprise, it did accelerate aggregation (Fig. 1g). We can thus conclude that although protein aggregation often precedes dormancy, the mere presence of protein aggregates is not sufficient to induce dormancy.

Protein aggregates progressively sequester proteins involved in translation, energy production and metabolism

Our observation that aggregation precedes dormancy in a wide variety of strains suggests that aggregation might induce dormancy in *E. coli*. However, the specific mechanism by which aggregation might induce dormancy is not known. It is hypothesized that the depletion of important proteins from the cytoplasm causes the shutdown of

pathways in which they are involved, thereby inducing dormancy. This hypothesis is supported by the observation that aggregates correlated with dormancy often contain proteins related to transcription, translation, metabolism and energy production^{19,24,25,33,37}. Moreover, reducing transcription^{52,53}, translation^{52,53} or energy levels⁵⁴ was demonstrated to induce persistence. Our objective was to explore if and how protein composition drives dormancy development.

To find key proteins that could induce dormancy upon aggregation, we assessed the composition of protein aggregates across the selected genetic backgrounds tested here. By using different strains, we filtered out proteins that only aggregate under specific conditions and that are therefore not generally associated with dormancy development. We grew all strains for 40 hours, to ensure they all exhibited dormancy (Fig. 1a, b and Supplementary Fig. 2), and then isolated their protein aggregates, which correspond to the insoluble fraction that is not resuspended by Triton X-100 and deoxycholate. Protein abundances were quantified by mass spectrometry (MS) analysis. Clusters of Orthologous groups (COG)⁵⁵ and Kyoto Encyclopedia of Genes and

Genomes (KEGG)⁵⁶ enrichment analyses were performed on the proteins identified from these aggregates to determine which protein classes were overrepresented (Fig. 2 and Supplementary Fig. 3a). These analyses revealed that all the strains contained a wide diversity of proteins (Supplementary Fig. 3a). Moreover, although all aggregates contained a wide diversity of proteins (Fig. 2a, b), they were all enriched in proteins associated with nucleotide transport and metabolism, translation, energy production and different metabolic processes (Fig. 2c, d), confirming previous results^{19,24,25,33,37}. Finally, aggregation occurs similarly in a strain overexpressing *era* and the vector control, with the exception of *Era* (Log2 fold change 7.76, $p = 0.019$).

Next, we analyzed if and how aggregate composition changes over time. To do so, we selected the strain with overexpression of *obgE* and the vector control strain for further analysis, as these two strains exhibit the most significant difference in aggregation dynamics. Moreover, extensive physiological and aggregation data are already available for these strains^{25,49,57,58}. We quantified for both strains their aggregate composition at different time points during growth using MS²⁵. In general, the aggregates contained a wide diversity of proteins with an enrichment in proteins involved in metabolism, energy production, nucleotide metabolism and transport, translation and post-translational modification, protein turnover and chaperone functions (Supplementary Fig. 3b–e). To specifically determine which protein classes were increasingly sequestered in the aggregates over time, we used the DESeq2 package⁵⁹ followed by a COG enrichment analysis⁵⁵ (Fig. 3). For the vector control strain, it is clear that aggregates sequester increasingly more proteins involved in energy production and translation over time (Fig. 3a). Moreover, in the strain overexpressing *obgE*, the number of proteins involved in energy production and translation are already high from the first time points sampled (16 and 24 hours) (Fig. 3b). This corresponds to what we expect based on the strong acceleration of dormancy and aggregation in this strain (Fig. 1b, d, f and Supplementary Fig. 2e, f). We can thus conclude that aggregates in dormant cells preferentially sequester proteins important for energy production and translation over time. The sequestration and depletion of these proteins could possibly shut down or reduce the corresponding processes and induce dormancy.

Protein aggregation drives dormancy development by triggering ATP depletion, but not by reducing translation

Because aggregates sequester proteins involved in energy production and translation over time, we investigated if the aggregation and depletion of these proteins might result in a lowering of energy production and translation, respectively, that could be linked to dormancy development. We therefore measured ATP depletion and translational activity over time and compared these measurements to the timing of aggregation and dormancy development. Single-cell ATP levels were quantified by ratiometric microscopy using the iATPSnFr^{1.0} sensor that changes its emission intensity after excitation at specific wavelengths based on ATP binding and therefore reports on the cellular ATP concentration¹⁸. At each time point under investigation, we determined the fraction of ATP-depleted cells as those cells that displayed an iATPSnFr^{1.0} ratio below a certain threshold (Fig. 4a and Supplementary Fig. 4). Additionally, as energy production is linked to membrane potential⁶⁰, we also measured membrane depolarization over time using bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC₄(3)), a fluorescent dye that exclusively penetrates depolarized cells⁶¹ (Fig. 4b). Finally, translation levels were determined using the Click-iT[®] HPG Alexa Fluor[®] Protein Synthesis Assay Kit (Thermo Fisher Scientific), which measures translation by monitoring the incorporation of an amino acid analog that can be fluorescently labeled through click chemistry (Fig. 4c). To better compare the timing of different processes, we plotted the moment at which the processes start and

reach their maximal level (Fig. 4d). Nonlinear logistic growth models were fitted to the data for IbpA and Ph aggregation, ATP depletion and membrane depolarization to determine both parameters (Figs. 1d, f, 4a, b and Supplementary Tables 5 and 6). The results of these dynamic measurements indicate that cells experience ATP depletion, membrane depolarization and a drop in translation in stationary phase (Fig. 4 and Supplementary Fig. 4). Moreover, we also observed that overexpression of *obgE* accelerates ATP depletion and membrane depolarization, suggesting a relation between them. In contrast, *ObgE* did not affect the drop in translation. As overexpression of *obgE* accelerates aggregation, dormancy, and energy depletion, but it does not decrease translation, we conclude that aggregation and dormancy are linked to a drop in the energy levels, but that there is no detectable relationship between aggregation, dormancy and the reduction in translation in our conditions.

Strikingly, when focusing on cellular energy levels, we observed that early-stage IbpA aggregation occurs before ATP depletion and membrane depolarization (Fig. 4d). This indicates that protein aggregation is not induced by ATP depletion and that the reduction in ATP levels is not directly caused by a reduced membrane potential. Furthermore, this suggests that the aggregation of proteins involved in energy production (between 32 and 72 hours for the vector control strain and between 8 and 16 hours for the strain overexpressing *obgE* (Fig. 3)) might induce ATP depletion (after 40 hours for the vector control strain and after 16 hours for the strain overexpressing *obgE*) and subsequently dormancy development. Importantly, the difference in timing of ATP depletion between the vector control strain and the strain overexpressing *obgE* cannot be explained by nutrient depletion alone as both strains reach stationary phase at approximately the same time (Supplementary Fig. 2). Surprisingly, our results also demonstrate that the energy levels in persister cells do not decline further when they transition to the VBNC state. This suggests that the decreased recovery of VBNC cells is not caused by a lower energy level, as suggested before^{22–26}, but that it might be influenced by other factors.

Delaying or accelerating protein aggregation affects dormancy similarly

To further support the hypothesis that protein aggregation induces dormancy, we manipulated protein aggregation by deleting or overexpressing the chaperone DnaK and monitored the effects on aggregation and dormancy development (Fig. 5a–h and Supplementary Fig. 5a–c). We observed that the deletion of *dnaK* indeed accelerates aggregation and advances the induction of dormancy in the cells (Fig. 5a–d and Supplementary Fig. 5a). Notably, while the deletion of *dnaK* leads to an earlier peak in persistence, the absolute number of persisters is generally lower in this strain (Fig. 5b). At the same time, there are significantly more VBNC cells, which also form earlier (Fig. 5c, d). These results suggest that the deletion of *dnaK* accelerates the induction of dormancy, but impedes the recovery and regrowth which is a defining feature of persisters.

Additionally, overexpression of DnaK is expected to reduce protein aggregation. Accordingly, we observe a significant decrease in Ph aggregation upon *dnaK* overexpression and a delayed dormancy development (Fig. 5e–h). However, we also detect an increase in IbpA aggregation at earlier time points (Fig. 5e and Supplementary Fig. 5b). This suggests that the IbpA aggregation observed is likely caused by the strong overexpression and partial aggregation of DnaK, which probably does not induce proteome-wide protein aggregation relevant to dormancy induction.

Thus, we conclude that inducing or preventing aggregation by altering cellular levels of the DnaK chaperone advances or delays dormancy development, respectively, further supporting the notion that protein aggregation contributes to the induction of dormancy.

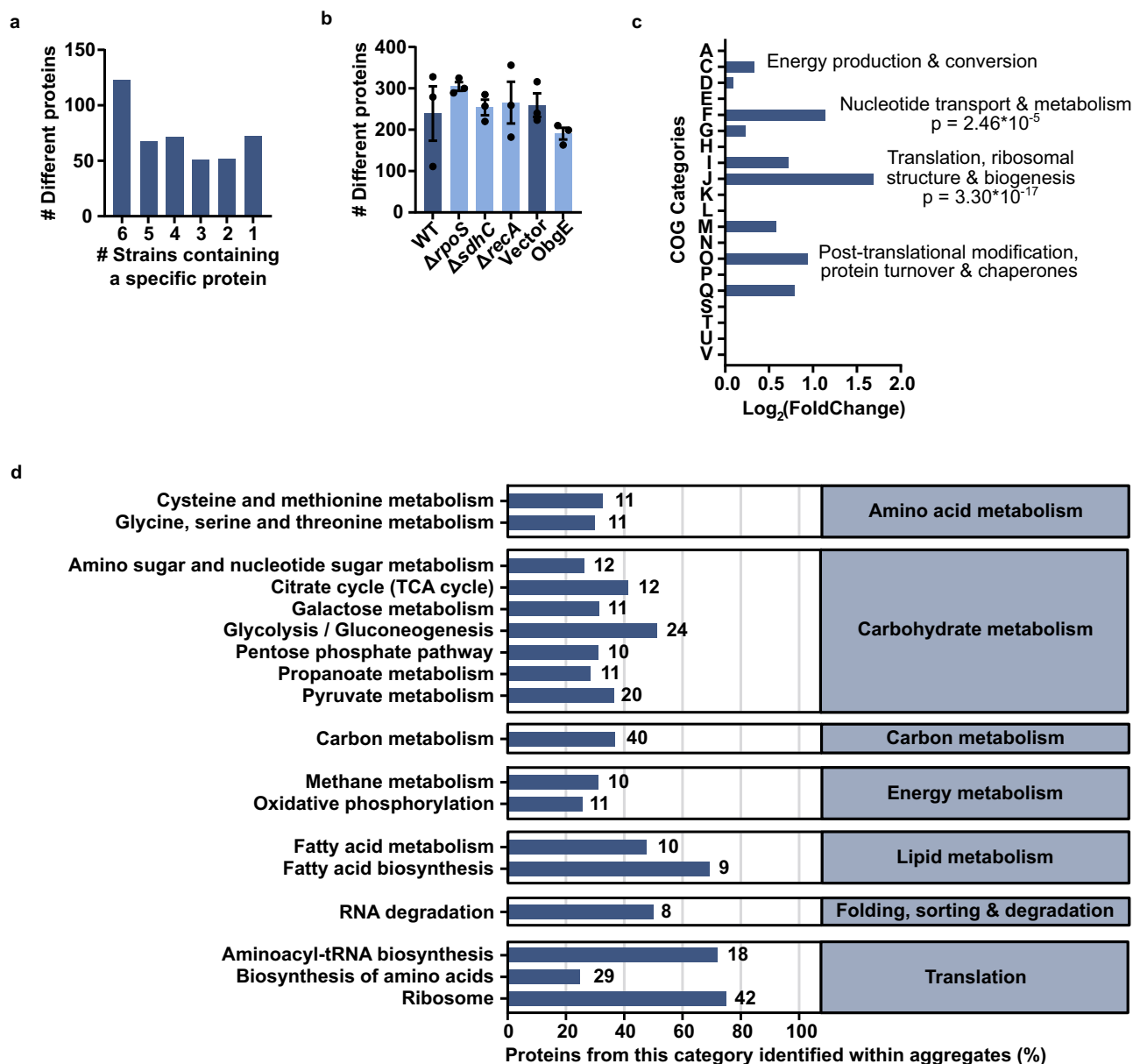


Fig. 2 | Aggregates preferably sequester proteins involved in energy production, translation and metabolism. Aggregate composition was quantified by MS using label-free quantitation values based on spectral counting after 40 hours of incubation for *E. coli* WT, *E. coli* $\Delta rpoS$, $\Delta sdhC$ and $\Delta recA$ and *E. coli* with pBAD33Gm (Vector) and pBAD33Gm-*obgE* (ObgE). **a** This graph shows the number of unique proteins that were aggregated in all 6 strains, in only one strain, and everything in between. **b** The number of unique aggregated proteins in each strain is indicated. Bar graphs and error bars represent the mean $\pm$ SEM ($n = 3$, with n the number of samples per strain from which the aggregates were purified). Mean and SEM for WT, $\Delta rpoS$, $\Delta sdhC$, $\Delta recA$, Vector and ObgE strains are 239.3 ± 65.71 , 304.7 ± 10.65 , 254 ± 18.81 , 265.7 ± 50.34 , 259.7 ± 28.59 and 190.7 ± 14.19 . **c** Aggregate composition data of all samples were combined to determine which COG categories were overrepresented using the one-sided Fisher's exact test with Bonferroni correction for multiple testing. **d** Aggregate composition data of all the strains were combined to determine which KEGG pathways were enriched using a hypergeometric test with the Benjamini-Hochberg multiple testing correction. Overrepresented

categories are shown ($P < 0.05$). Exact p -values are (top to bottom): 0.0024, 0.0041, 0.0067, 0.00026, 0.0029, 1.01×10^{-8} , 0.0046, 0.0057, 1.16×10^{-5} , 3.41×10^{-10} , 0.0046, 0.0104, 0.00036, 8.72×10^{-5} , 0.0096, 1.58×10^{-8} , 6.73×10^{-5} and 8.35×10^{-19} . The absolute number of aggregated proteins that belong to each category is indicated next to the bars. COG categories represented in **c** are: A—RNA processing and modification, C—energy production and conversion, D—cell cycle control, cell division, and chromosome partitioning, E—amino acid transport and metabolism, F—nucleotide transport and metabolism, G—carbohydrate transport and metabolism, H—coenzyme transport and metabolism, I—lipid transport and metabolism, J—translation, ribosomal structure, and biogenesis, K—transcription, L—replication, recombination, and repair, M—cell wall/membrane/envelope biogenesis, N—cell motility, O—posttranslational modification, protein turnover, and chaperones, P—inorganic ion transport and metabolism, Q—secondary metabolites biosynthesis, transport, and catabolism, S—function unknown, T—signal transduction mechanisms, U—intracellular trafficking, secretion, and vesicular transport, V—defense mechanisms. Source data are provided as a Source Data file.

DnaK is important for the recovery and regrowth of dormant cells

After providing strong indications that protein aggregation induces dormancy, we wondered whether aggregates need to be removed before the cells restart growth. We observed that some cells

overexpressing *obgE* spontaneously induce disaggregation and recovery from dormancy in late stationary phase (Fig. 1d, f and Supplementary Fig. 2f) and that this disaggregation precedes the increase in CFUs we observe in late-stationary phase (Supplementary Fig. 5d). We verified that the spontaneous increase in CFUs after

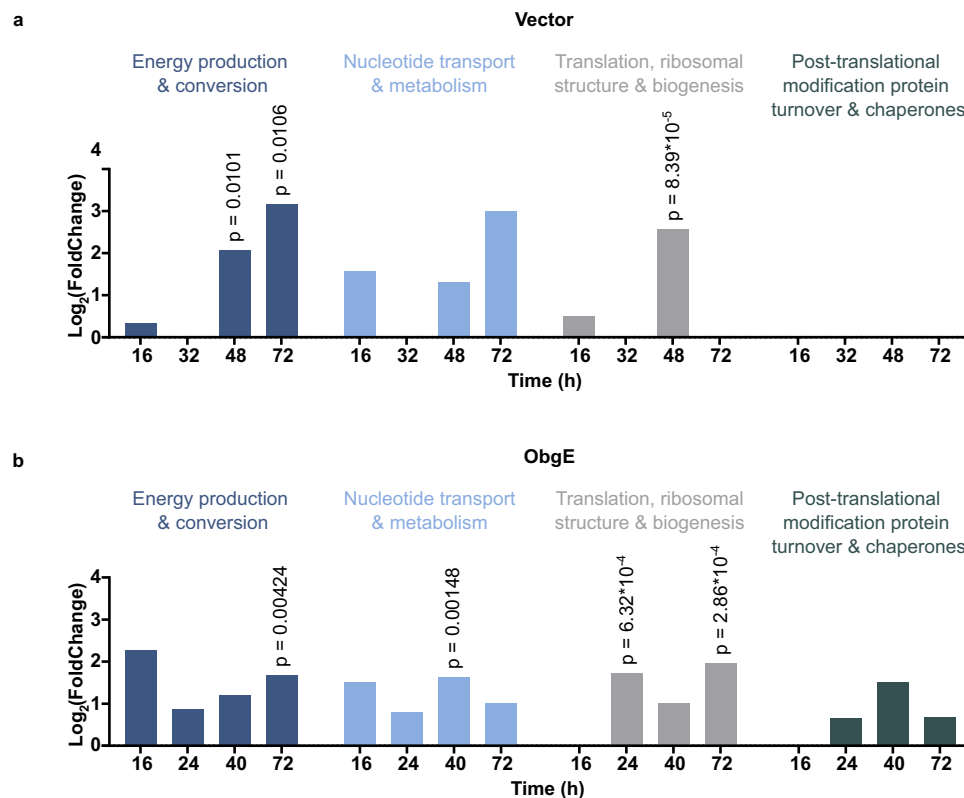


Fig. 3 | Aggregates progressively sequester proteins involved in energy production and translation. Aggregates were isolated and their composition was quantified for *E. coli* pBAD33Gm (Vector) (a) after 8, 16, 32, 48 and 72 hours of incubation and for *E. coli* pBAD33Gm-*obgE* (ObgE) (b) after 8, 16, 24, 40 and 72 hours of incubation ($n = 4$ for V32 and $n = 5$ for all the other samples). For the last

4 sampling points, protein abundances in the aggregate were compared to the abundances in the previous sampling point to identify enriched proteins and their COG categories using the one-sided Fisher's exact test with Bonferroni correction for multiple testing. Source data are provided as a Source Data file.

overexpression of *obgE* was due to real recovery and not due to the growth of mutants that have obtained the growth advantage in stationary phase (GASP) phenotype⁶² (Supplementary Table 8). This implies that cells overexpressing *obgE* can spontaneously cause disaggregation and thereby recover from the VBNC state, leading to an increase in CFUs. This is consistent with previous observations showing that persister cells remove their aggregates before they restart growth^{24,25}.

As it was previously demonstrated that the chaperone DnaK plays an important role during disaggregation and recovery^{24,38}, we wanted to expand this investigation to other chaperones and proteases. We therefore deleted the most important chaperones and proteases in *E. coli* and measured their effects on the number of CFUs and viable cells after 72 hours of incubation (Fig. 5i). While none of the deletions significantly alter the number of viable cells, the deletion of *dnaK* significantly decreases the number of culturable cells (i.e. CFUs), indicating inhibition of recovery due to lower disaggregation. Remarkably, deleting proteases or other chaperones does not significantly affect the number of CFUs after 72 hours, although we did see non-significant decreases for many deletion strains. These data illustrate that, besides DnaK, none of the chaperones or proteases tested play a crucial role individually in disaggregation, even though they may be of minor assistance in the process. These results underscore that cellular regrowth and exit from dormancy is mainly dependent on DnaK. DnaK is known to function in disaggregation and subsequent refolding of disaggregated proteins⁶³, indicating that refolding of liberated proteins might be important for recovery. Indeed, 40 to 50 percent of the aggregated proteins were identified as DnaK interactors (Fig. 5j), while only 25 percent of the cytosolic proteome interacts with DnaK⁶⁴. The high percentage of DnaK interactors

in the aggregates supports the idea that DnaK-dependent refolding is important during recovery and regrowth.

Proteins first assemble into liquid-like condensates, that develop into solid aggregates over time

We provided support for the hypothesis that aggregation induces dormancy by demonstrating that aggregation precedes ATP depletion and dormancy, but also by showing that aggregates are removed before the cells recover from their dormant state. As VBNC cells have a much lower regrowth capacity compared to persister cells, we hypothesized that a difference in disaggregation potential could be responsible. The disaggregation potential of aggregates is strongly affected by the aggregate structure⁶⁵. Indeed, it was observed previously in eukaryotic cells that proteins often first assemble into liquid-like structures that subsequently progress into a more solid-like end-stage of aggregation which is less easily reversible^{40,65}. The liquid-like structures that remain easily reversible are referred to as biomolecular condensates, whereas the irreversible solid-like structures are termed aggregates⁶⁶. Therefore, we verified whether the early-stage IbpA aggregates and the late-stage Ph aggregates, which correlate with persisters and VBNC cells, respectively, are indeed true aggregates or if they are biomolecular condensates with liquid-like properties²⁵.

To determine the aggregate structure and solubility, strains overexpressing *obgE* were grown for 8 or 40 hours, to obtain cells mainly associated with early-stage IbpA foci and cells containing further developed Ph foci, respectively (Fig. 1d, f). The IbpA and Ph foci within these cells were imaged on agar pads containing 1,6-hexanediol (Fig. 6a–c and Supplementary Fig. 6a, b) as this chemical is known to only dissolve liquid-like condensates and not solid-like aggregates⁶⁷. Quantitative microscopy analysis revealed that the fraction of cells

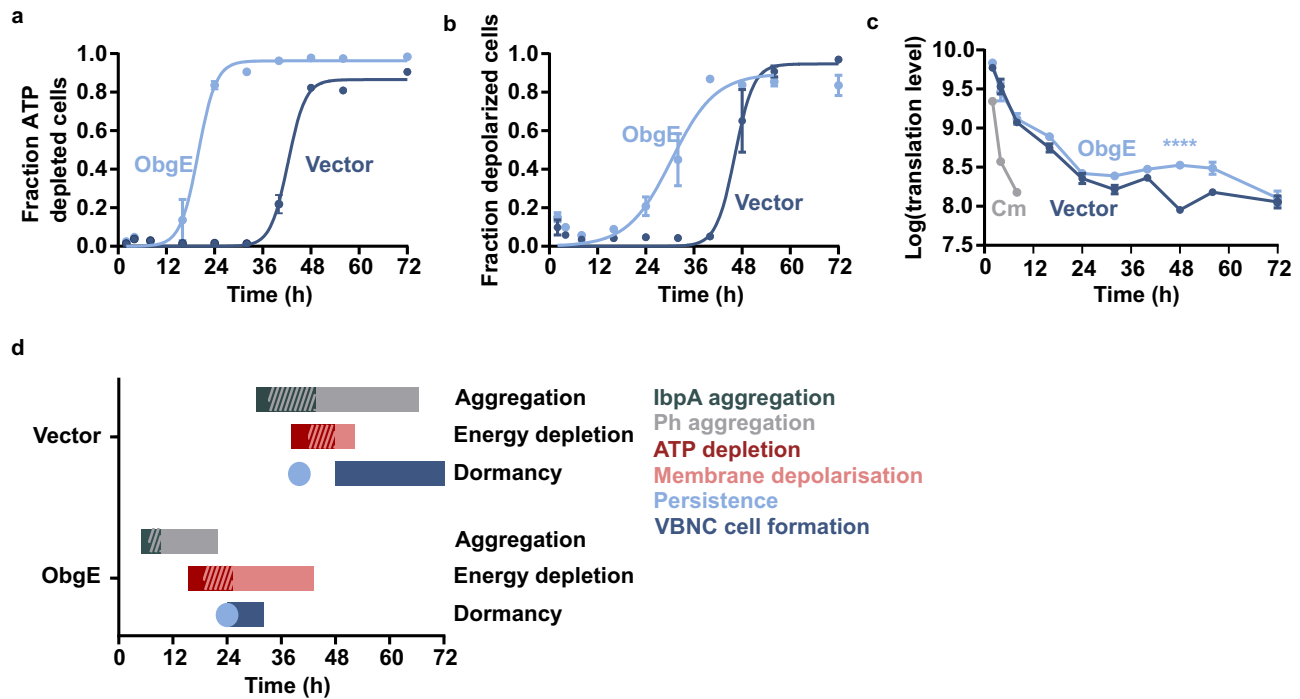


Fig. 4 | Early-stage lbpA aggregation precedes ATP depletion, membrane depolarization and VBNC cell formation in stationary phase. All measurements were performed with *E. coli* pBAD33Gm (Vector) and pBAD33Gm-*obgE* (ObgE). **a** Based on the single-cell ATP measurements and the threshold for ATP depletion indicated in Supplementary Fig. 4, the fraction of cells with ATP depletion was determined at different time points. A nonlinear logistic growth model was fitted onto the data (solid line). **b** The fraction of cells with membrane depolarization was determined at different time points by measuring the proportion of the bacterial population that stained with DiBAC₄(3), a fluorescent dye that penetrates depolarized cells. A nonlinear logistic growth model was fitted onto the data (solid line). **c** Translation was measured using the Click-iT[®] HPG Alexa Fluor[®] Protein Synthesis Assay Kit. As a negative control, the Vector was treated with the translation

inhibitor chloramphenicol (Cm). For each repeat and each time point, the sum of the fluorescence intensity in 10,000 bacterial cells is shown. Translation levels were compared between the Vector and ObgE at the different time points by unpaired two-sided *t*-tests with the Holm-Šidák correction for multiple comparisons (for the comparison after 48 hours of incubation: *****P* = 0.000082). Data in graph a–c are represented as averages ± SEM, (*n* = 4) (See Supplementary Table 7 for exact values). **d** The timing of dormancy (persistence and VBNC cell formation), energy depletion (ATP depletion and membrane depolarization) and aggregation (lbpA and Ph aggregation) is represented. For persistence, the peak moment is indicated, while for the other processes, both the initiation and the peak moment are shown. Source data are provided as a Source Data file.

containing lbpA foci after 8 hours of incubation decreased more than 3-fold in the presence of hexanediol (Fig. 6a). Dissolution of lbpA foci was also clear from the fluorescence pattern of lbpA-msfGFP transitioning from distinct foci to a more diffuse distribution (Fig. 6c). Importantly, the small number of Ph foci that were already present after 8 hours of incubation could not be dissolved by hexanediol, suggesting that only early-stage lbpA foci have liquid-like properties (Supplementary Fig. 6a). Indeed, after 40 hours of incubation, most lbpA foci were associated with Ph foci (Fig. 6c) and these Ph foci could not be dissolved by hexanediol (Fig. 6b and Supplementary Fig. 6b). The same was found to be true for a *dnaK* deletion strain (Fig. 6a, b and Supplementary Fig. 6a, b), indicating that the intrinsic solubility of the aggregates is independent of DnaK. This indicates that early-stage lbpA foci show liquid-like properties, while late-stage Ph structures are more solid-like.

To validate the increased solidification of aggregates over time, we performed FRAP (fluorescence recovery after photobleaching) on a section of lbpA-msfGFP-stained foci in cells that were incubated for 8 (early-stage aggregates) or 40 (late-stage aggregates) hours. In this procedure, a small piece of the aggregate was bleached and recovery of fluorescence inside the aggregate was monitored over time (Fig. 6d–f). Early-stage lbpA foci recovered approximately 35% of their fluorescence intensity after bleaching over a time period of 50 seconds with a half time of 15.4 ± 22.7 seconds, indicating protein mobility, which is a characteristic of liquid-like condensates (Fig. 6d, f and Supplementary Fig. 6c). The low percentage of recovery can probably

be attributed to the large fraction of the lbpA focus that was bleached, owing to the small size of the bacteria. However, another explanation might be that the condensates exhibit gel-like properties instead of liquid-like. Since the distinction between liquid-like and gel-like condensates is not yet clear and considering that the low recovery is likely due to extensive bleaching, we still anticipate the condensates to behave as liquid-like. In contrast to the early-stage lbpA foci, late-stage lbpA foci did not display recovery (<1%) (Fig. 6e), confirming that liquid-like, early-stage condensates solidify over time into aggregates.

Finally, we investigated whether the transition from liquid-like condensates to solid-like aggregates also involves structural changes in proteins. We therefore investigated the structure of both early- and late-stage aggregates in *E. coli* cells overexpressing *obgE* using atomic force microscopy-based infrared spectroscopy (AFM-IR). AFM-IR spectroscopy combines the subwavelength resolution of AFM microscopy with IR spectroscopy, to obtain chemical and structural information on the sample under investigation⁶⁸. Additionally, analysis of the amide I band in IR spectra offers information about the secondary structure of proteins⁶⁹. For our analysis, we incubated *E. coli* pBAD33Gm-*obgE* for 2, 8 and 40 hours to obtain cells with no, early-stage condensates and late-stage aggregates, respectively (Fig. 1d, f). For each sample, we measured the IR absorbance at 1625 cm^{-1} to estimate the abundance of β -sheets in the cells⁶⁹ (Fig. 6g–i and Supplementary Fig. 6d–i). The images obtained after 2 and 8 hours of incubation show a homogeneous absorbance within the cells. However, images measured after 40 hours of incubation show specific

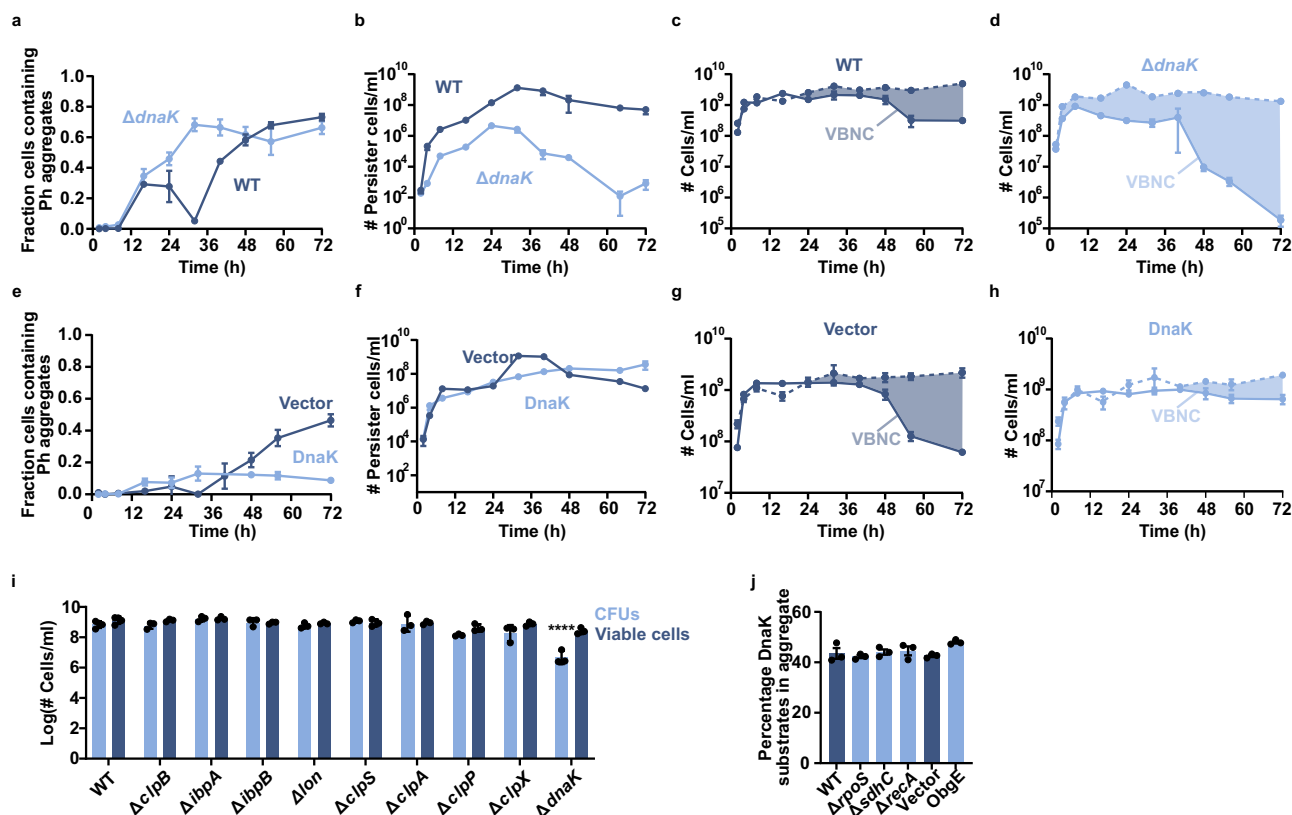


Fig. 5 | DnaK decreases dormancy and is important for regrowth. Aggregation, persistence and VBNC cell formation were measured for *E. coli* WT, *E. coli* Δ dnaK (a–d), *E. coli* pBAD33Gm (Vector), and *E. coli* pBAD33Gm-dnaK (DnaK) (e–h). **a, e** Quantitative analysis of microscopy images shows the fraction of cells containing Ph aggregates. Data are represented as averages $\pm$ SEM, ($n \geq 3$, > 50 cells analyzed for every repeat) (Supplementary Tables 2–4 and 9–12 for exact values). **b, f** Persistence was measured at different time points. Data are represented as averages $\pm$ SEM, ($n \geq 4$) (Supplementary Tables 2–4 and 9–12 for exact values). **c, d, g, h** The number of CFUs (solid line) and viable cells (dashed line) were measured at different time points. The area between both curves represents the number of VBNC cells. Data are represented as averages $\pm$ SEM, ($n \geq 4$) (See Supplementary Tables 2–4 and 9–12 for exact values). Note that viable cells and CFUs are measured differently and are therefore not perfectly comparable. The discrepancy observed at some early timepoints suggests that the number of viable cells may be underestimated. **i** The number of CFUs and viable cells was determined after 72 hours of *obgE* overexpression in WT and deletion strains. The logarithm of the number of viable and culturable cells was compared between WT and deletion strains using a two-way ANOVA with Dunnett's correction for multiple comparisons (Δ dnaK: q ratio = 9.771, 42 degrees of freedom, **** $P < 0.0001$). Bar graphs and error bars represent averages $\pm$ SEM, ($n \geq 3$) (See Supplementary Table 13 for exact values). **j** The percentage of proteins that are aggregated after 40 hours of incubation and that are a substrate of DnaK was calculated⁶⁴. Bar graphs and error bars represent averages $\pm$ SEM, ($n = 3$, with n the number of samples per strain from which the aggregates were purified). For WT, Δ rhoS, Δ sdhC, Δ recA, Vector and ObgE strains, the mean $\pm$ SEM values are 43.56 ± 2.123 , 42.27 ± 0.5844 , 44.07 ± 1.067 , 44.55 ± 1.785 , 42.59 ± 0.4752 and 48.01 ± 0.5392 . Source data are provided as a Source Data file.

regions with a high absorbance at 1625 cm^{-1} , which points at a high β -sheet content and the presence of aggregates. To get more structural information, we also selected different points for which we measured the full IR spectrum with a focus on the amide I ($1600\text{--}1700\text{ cm}^{-1}$) and amide II ($1500\text{--}1600\text{ cm}^{-1}$) bands (Fig. 6g–i and Supplementary Fig. 6d–i). These results show that the spectra taken after 2 hours of incubation and most of the spectra taken after 8 hours of incubation are all quite similar and do not have a high absorbance at 1625 cm^{-1} , further confirming that these cells are quite homogeneous with a low β -sheet content. However, after 40 hours of incubation and to a lesser extend after 8 hours of incubation, spectra taken at regions with a high 1625 cm^{-1} absorbance display a shoulder in the amide I band spectrum at 1625 cm^{-1} , which is indicative for the presence of intermolecular β -sheets. Indeed, when we quantify the number of β -sheets relative to α -helices from the different spectra, we see a clear split between points with high and low β -sheet content after 40 hours of incubation, but not so much after 2 and 8 hours of incubation (Fig. 6j). Because cells that were incubated for 8 hours, and that were shown to contain early-stage condensates, show a rather homogeneous absorption of the amide I band within the cells, we can conclude that their condensates do not contain clusters with many β -sheets. However, as a few spectra already

show a shoulder in the amide I band spectrum at 1625 cm^{-1} , there seems to be an early transition to the more β -rich aggregates in a few cells at this time point. On the contrary, cells that were incubated for 40 hours, and that are characterized by late-stage aggregates, show a very high abundance of intermolecular β -sheets within their aggregates. Overall, these data demonstrate that the number of intermolecular β -sheets within aggregates increases during their maturation. The increased number of intermolecular interactions agrees with the observation that mature aggregates have a more solid-like structure and a less efficient dissolution. This phase transition from liquid-like condensates to solid-like aggregates over time is likely to hinder disaggregation, thereby explaining why growth resumption in VBNC cells, which are associated with late-stage aggregates, is challenging. This implies that the transition from persistence to the VBNC state is controlled by a reduced recovery due to solidification of protein condensates.

Discussion

Dormant persister and VBNC cells have both been associated with protein aggregation^{19,24,25,34,37,38}. We delved deeper into the relationship between dormancy and aggregation and provide support for the

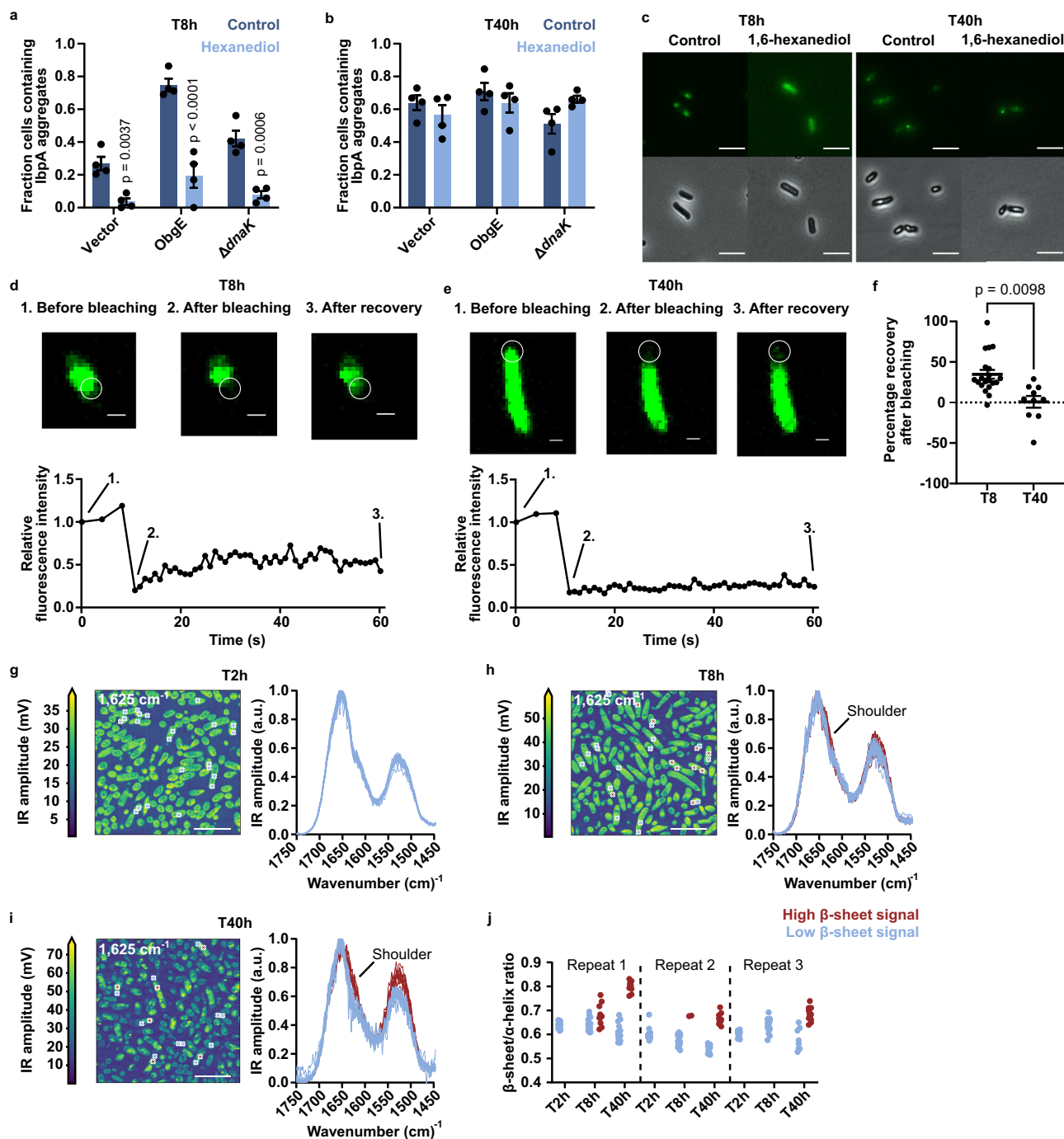


Fig. 6 | Early-stage condensates show liquid-like properties, while solidified late-stage aggregates contain more intermolecular β -sheets. After 8 (a, c) and 40 (b, c) hours of incubation, *E. coli* *ibpA-msfGFP* with pBAD33Gm (Vector) or pBAD33Gm-*obgE* (ObgE) and *E. coli* *ibpA-msfGFP* Δ *dnaK* were imaged with and without 10% 1,6-hexanediol. Quantitative microscopy analysis shows the fraction of cells with IbpA foci in each condition. For the Vector, ObgE and Δ *dnaK* strains, the fraction of cells with IbpA foci was compared between cells treated with and without hexanediol using a two-way ANOVA with Holm-Šidák correction for multiple comparisons. Bar graphs and error bars represent averages $\pm$ SEM, ($n = 4$, ≥ 50 cells were analyzed for each repeat) (see Supplementary Table 13 for exact values). **c** Representative microscopy images are shown. Scale bar, 5 μm . FRAP was performed on a small part of the IbpA foci of *E. coli* *ibpA-msfGFP* pBAD33Gm-*obgE* after 8 (d) and 40 (e) hours of incubation. Representative images before and right after bleaching, and after recovery are presented as well as the relative fluorescence

compared to the starting point. Scale bar, 1 μm . **f** The percentage recovery after bleaching is shown. The recovery was compared using a two-sided Wilcoxon signed-rank test. Data are represented as averages $\pm$ SEM. *E. coli* pBAD33Gm-*obgE* was incubated for 2 (g), 8 (h) and 40 (i) hours and 3 repeats of the samples were imaged using AFM-IR spectroscopy. The results of the first repeat are presented here. The IR absorbance at 1625 cm^{-1} for the complete sample is shown on the left. Scale bar, 5 μm . Different foci within the sample were selected for which a full IR spectrum was measured (indicated by crosses). The complete IR spectra of these foci are shown on the right. k-means clustering on the second derivative of the amide I band (1580 cm^{-1} –1700 cm^{-1}) was performed to discriminate between spectra with high and low β -sheet signal (red and blue, respectively). **j** For each complete IR spectrum (Fig. 6g–i and Supplementary Fig. 6d–i), the fraction of β -sheets compared to α -helices was determined. Source data are provided as a Source Data file.

hypothesis that aggregation might induce dormancy in *E. coli*. First, we demonstrate that protein aggregation always precedes dormancy development during starvation in a variety of strains displaying diverse dormancy dynamics (accelerated or delayed). Second, we confirm that aggregates are enriched in proteins involved in translation, energy production and metabolism. Moreover, we show that the sequestration of proteins active in energy production precedes—and therefore likely induces—ATP depletion and dormancy. Third, we establish that either delaying or accelerating aggregation by changing the levels of the chaperone DnaK also affects dormancy similarly. Fourth, we confirm that aggregates need to be removed before dormant cells can start regrowth^{24,25,37,38}. Importantly, we also show that the efficiency of aggregate dissolution is dependent on the presence of the chaperone DnaK^{24,38}, but also on the structure of the aggregates. More specifically, persister cells are associated with early-stage condensates that are easily removed because of their liquid-like structure. On the other hand, VBNC cells often contain late-stage aggregates that have matured to obtain a solid-like structure, probably because of their increased number of intermolecular β -sheets, which impedes their disaggregation. Based on these results, we propose a model that explains how aggregation might induce dormancy (Fig. 7). As bacteria experience starvation, they progressively sequester more proteins that are active in energy production into protein aggregates. This aggregation and depletion of protein function shuts down the energy production pathway and causes ATP depletion. This energy depletion then induces the onset of dormancy and antibiotic tolerance. These results correspond to earlier observations that persister cells have lower ATP levels^{18–20}. Proteins assemble into liquid-like condensates in the early stages of development, which allows them to be dissolved easily so that dormant cells can quickly recover and restart growth. However, over time these condensates solidify into aggregates. As it is hypothesized that ATP-driven remodeling is needed to keep the condensates in a liquid-like structure⁷⁰, the low levels of cellular ATP throughout stationary phase might ultimately cause this aggregate solidification. Solidified aggregates significantly hinder disaggregation and this slower disaggregation complicates the recovery from dormancy and pushes the persister cells into the VBNC state. Although solidified protein aggregates cannot be easily dissolved, it

has been reported that they can be converted back to the liquid-like state after which dissolution and presumably regrowth can take place⁶⁵. This conversion from a solid-like to a liquid-like structure was shown to be catalyzed by chaperones when the aggregation-inducing stress was removed⁶⁵. Although this study focused on the over-expression of firefly luciferase⁶², which is difficult to refold, we hypothesize that a similar process might also solubilize endogenous proteins from the aggregates and thereby induce recovery and regrowth of VBNC cells generated under our conditions. No conditions were found yet under which the solid-like aggregates in the VBNC cells are solubilised. Additionally, the (apparent) irreversibility of VBNC solid aggregates versus the reversibility of persister states is in agreement with the model of phase separation coupled to percolation⁷¹. In this model, irreversible aggregation by percolation is characterized by a networking transition where individual molecules or clusters form a network that spans the entire system once a critical concentration, known as the percolation threshold, is reached (c_{perc}). If the saturation concentration for phase transition is lower than c_{perc} (reversible) phase separation occurs first, and the dense phase itself can subsequently undergo percolation to form a network structure (microgel).

We here provide support for the hypothesis that aggregation induces dormancy. However, it is important to note that although aggregation precedes dormancy in various *E. coli* strains, the mere presence of aggregates is not always sufficient to trigger dormancy. We showed that aggregates progressively sequester proteins involved in energy production and that this is followed by ATP depletion and dormancy. This indicates that aggregate composition is an important factor in determining the influence of aggregation on dormancy. We therefore hypothesize that aggregates might only induce dormancy when they sequester a certain threshold of proteins active in energy production. Additionally, we only investigated the relation between aggregation and dormancy in *E. coli*, which means that these results need to be confirmed in other strains to draw general conclusions about the role of protein aggregation in bacterial dormancy. Results of other groups on the relation between aggregation and dormancy in *Staphylococcus aureus* suggest that this link may indeed be valid across multiple species^{19,31}.

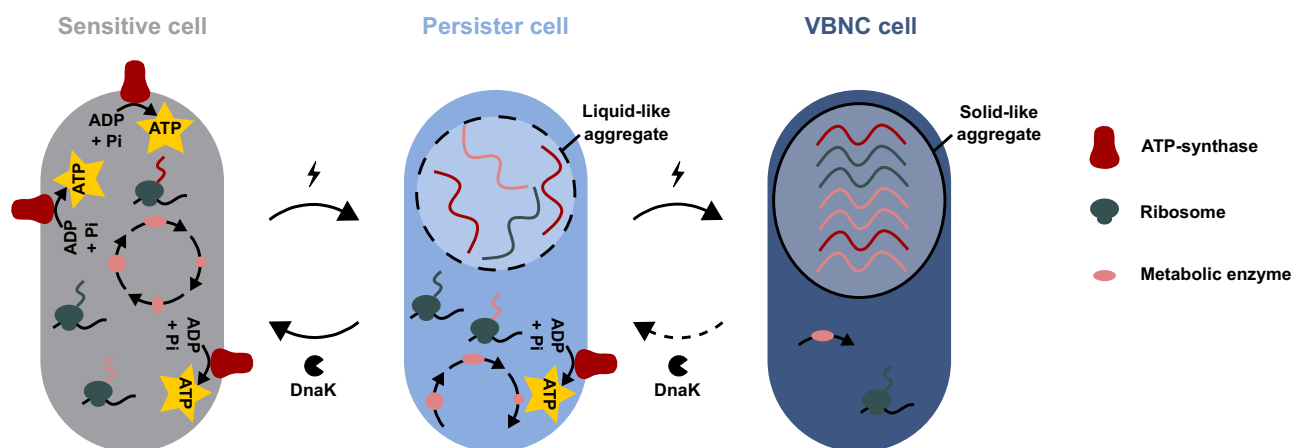


Fig. 7 | Model describing how protein aggregation induces different categories of dormancy. In response to stress such as nutrient exhaustion, bacteria progressively sequester proteins in liquid-like condensates. The condensation of proteins active in energy production precedes—and thereby likely induces—a depletion of cellular ATP and dormancy. The chaperone DnaK is able to dissolve these condensates due to their liquid-like structure. This dissolution allows the recovery and regrowth of the persister cells. Restart of growth is supported by dissolved and refolded proteins that are released from the biomolecular condensate. Indeed, the condensates in dormant cells contain a variety of proteins

involved in translation, energy production and metabolism which could be repurposed for growth. However, if stress persists over an extended period of time, the liquid-like condensates solidify into aggregates by accumulating intermolecular β -sheet structures. This transition complicates their removal by DnaK and consequently the exit from dormancy. The solidification of the aggregates thereby pushes the shallowly dormant persister cells in the deeper dormant VBNC state. Under specific conditions, solidified aggregates from VBNC cells can presumably be converted back to liquid-like structures (dashed arrow), which is thought to allow disaggregation by DnaK and subsequent regrowth.

Furthermore, we provide deeper insight into the difference between persister and VBNC cells. Persister cells were hypothesized to transition to the VBNC state by lowering their energy levels and/or metabolic activity^{22–26}. Our data do not support this hypothesis as we observed that persister and VBNC cells have similar energy levels. Rather our results suggest that the shift from persistence to the VBNC state is primarily the result of aggregate solidification which determines the cell's potential to recover and restart growth.

When investigating the association between aggregation and dormancy, we discovered that these processes are very dynamic. Persister levels fluctuate significantly over time and persister cells are believed to shift to the VBNC state²⁵. Not measuring the dynamics of these processes in experiments could potentially lead to incorrect conclusions. As an example, the deletion of *sdhC* was previously claimed to decrease both aggregation and persistence in stationary phase^{35,37}. However, our measurements indicate that this deletion does not decrease these processes, but rather delays them. We therefore urge to change the assays for antibiotic tolerance from a one-point measurement to a dynamic measurement of both persistence and VBNC cell formation.

In addition to persister cells³⁵, many eukaryotic cells have also been associated with liquid-like condensates such as processing bodies, stress granules or aggregates induced by heat or nutrient stress^{72–74}. These initially liquid-like condensates are formed upon stress and also solidify over time^{73,75,76}. They serve as storage structures since they sequester numerous mRNA molecules, as well as translation- and metabolism-related proteins, and protect them from degradation^{77–81}. Before regrowth, disaggregation takes place and most of the molecules inside these liquid-like condensates are reused for a rapid restart of growth^{77–81}. The aggregates of dormant bacteria share various similarities with these eukaryotic storage structures. We not only demonstrate that condensates of dormant bacteria are liquid-like and solidify over time into aggregates, but also that they are enriched in proteins important for translation, energy production and metabolism. Additionally, regrowth of dormant cells was shown to strongly depend on DnaK but not on any of the other chaperones and proteases tested (ClpB, IbpA, IbpB, Lon, ClpS, ClpA, ClpP and ClpX). DnaK is known to be important for the refolding of proteins, while the tested proteases help with degrading misfolded proteins after disaggregation⁶³. Moreover, we observed that many of the aggregated proteins interact with DnaK for folding⁶⁴. These findings are in agreement with the hypothesis that the aggregated proteins are refolded by DnaK and reused instead of being degraded after aggregate dissolution³⁷. We therefore hypothesize that the liquid-like condensates in persister cells also serve as a storage structure to fuel a fast recovery and regrowth (Fig. 7).

Methods

Bacterial strains and growth conditions

All experiments were performed with *E. coli* BW25113⁸². To measure protein aggregation, *E. coli* BW25113 *ibpA-msfGFP*²⁵ was used while the *E. coli* BW25113 *iATPSnFr*^{1.0} strain was used for ATP measurements. The *E. coli* strain with genomic insertion of *iATPSnFr*^{1.0} was constructed by first transforming *E. coli* BW25113 with the pGRG25-*iATPSnFr*^{1.0} plasmid¹⁸ and then integrating the gene in the *attTn7* site⁸³. To obtain the Δ *rpoS*, Δ *sdhC*, Δ *dnaK* and Δ *recA* knockout strains, we started from the corresponding deletion strains in the Keio collection⁸². The kanamycin resistance cassette within these knockout strains was removed using the pCP20 plasmid⁸⁴.

The plasmids pBAD33Gm-*obgE*⁸⁵ and pBAD33Gm-*era* were used to induce overexpression of *obgE* and *era*. The empty vector pBAD33Gm⁸⁵ served as a control in all these measurements. The pBAD33Gm-*era* was constructed by replacing the *obgE* gene in the pBAD33Gm-*obgE* plasmid by the *era* gene via Gibson assembly (BioLabs).

For this Gibson assembly, the plasmid was amplified with the primers SPI13781 and SPI13782 while the *era* insert was amplified from a wild-type *E. coli* BW25113 strain with the primers SPI13779 and SPI13780. Verification of the right insert in the final plasmid was conducted by sequencing with primers SPI10499 and SPI10500. Sequences of the primers are SPI10499: CTACTGTTTCTCCATACCCG, SPI10500: TGTTTATCAGACCGCTTCT, SPI13779: CCGCCAAAACAGCCAAAGCTTTTA AAGATCGTCAACGTAACCGAGACT, SPI13780: GAGCTCAGGAGGAAT-TAACCATGAGCATCGATAAAAGTTACTGCGG, SPI13781: GTTACGTTG ACGATCTTTAAAAGCTTGGCTGTTTTGGCG, SPI13782: TAACCTTT ATCGATGCTCATGGTTAATTCCTCCTGAGCTCGAAT.

Bacteria were cultivated in lysogeny broth (LB) at 37 °C under orbital shaking. When the strains carried plasmids, the antibiotics to which these plasmids conferred resistance (e.g. 25 µg/ml gentamycin, 40 µg/ml kanamycin, 100 µg/ml ampicillin) were added to the growth medium. Overnight cultures were diluted 100-fold in flasks containing 100 ml LB, the appropriate antibiotics and 0.2% arabinose for strains carrying plasmids. At designated time points, a sample was taken from the flasks and analyzed. For translation measurements, cells were also shortly incubated in M9 minimal medium with 1% casamino acids.

Persister assay

To determine the number of persister cells at a specific time point, 990 µl of culture was transferred from a culture flask to a sterile test tube and treated with 5 µg/ml ofloxacin for 5 hours. Fluoroquinolone antibiotics are regularly used to clear infections of *E. coli*^{86,87}. Ofloxacin was chosen as it is often used in the persister field^{13,25,88,89}. After the antibiotic treatment, the cells were washed twice with 10 mM MgSO₄. 10-fold dilutions were prepared in 10 mM MgSO₄ and plated out on non-selective LB plates containing 1.5% agar. The number of CFUs was determined after incubation at 37 °C for 48 hours.

Identifying the number of VBNC cells

The number of VBNC cells was determined by measuring the difference between the number of viable cells and the number of culturable cells at a specific time point, similar to what was described before²⁵. Viable cells were quantified by staining the cells prior to antibiotic treatment with SYTOX Green (Invitrogen), which is a fluorescent and membrane-impermeable dye that can only penetrate dead cells that have lost membrane integrity. Cells were diluted 100- to 1000-fold in 0.5 µM SYTOX Green in filtered phosphate-buffered saline (PBS) and stained for 15 minutes in the dark. Subsequently, cells were measured with the CytoFLEX S flow cytometer (Beckman Coulter Life Sciences) equipped with 405-nm, 488-nm and 561-nm lasers. The forward and side scatter were used to gate bacterial cells (Supplementary Fig. 7). Fluorescence from the SYTOX Green dye was measured for 10,000 bacterial cells at an excitation wavelength of 488 nm and an emission wavelength of 525/40 nm. The number of viable cells per ml was calculated by counting the number of non-fluorescent, living cells per ml.

The number of culturable cells was identified by assessing the number of CFUs per ml of a culture that was not treated with antibiotics. 10-fold dilutions of the cultures were made in 10 mM MgSO₄ and plated on LB with 1.5% agar. The CFUs were counted after 24 hours of incubation at 37 °C and the number of CFUs per ml was calculated.

Measuring aggregation

The fraction of cells containing protein aggregates was measured by quantitative microscopy as described before²⁵. For all experiments, a Nikon Ti-E inverted microscope equipped with a Qi2 CMOS camera and temperature-controlled cage incubator was used. Cells were collected at different time points, spotted on pads of 10 mM MgSO₄ with 2% agarose and imaged in both the green fluorescent and the phase contrast channel. The measurement of the fraction of cells with Ph

aggregates in the *E. coli* *ibpA-msfGFP* pBAD33Gm-*dnaK* strain after 40 hours of incubation was deleted from Fig. 5e because it was an outlier that could be attributed to cells temporarily becoming a little lighter at mid-cell position (Supplementary Fig. 5c). All datapoints of Ph aggregation for the *E. coli* *ibpA-msfGFP* pBAD33Gm-*dnaK* strain and of the *E. coli* *ibpA-msfGFP* pBAD33Gm strain are shown. To test the dissolution of aggregates by hexanediol, the same culture was also spotted on pads of 10 mM MgSO₄ with 10% 1,6-hexanediol and 2% agarose. IbpA aggregates were determined as foci in the green fluorescent channel, while Ph aggregates appeared as white foci in the phase contrast channel. The ImageJ plug-in MicrobeJ was used for the image analysis⁹⁰. For each repeat, at least 50 cells were analyzed and the number of IbpA and Ph aggregates was determined in each cell. Based on these results, we calculated the fraction of cells that contained one or more aggregates.

Measuring ATP levels

E. coli BW25113 cells with the genomic iATPSnFr^{1.0} sensor were used to measure ATP at the single-cell level¹⁸. At specific time points, cells were diluted 100- to 1000-fold in filtered PBS and measured with the CytoFLEX S flow cytometer (Beckman Coulter Life Sciences) containing 405-nm, 488-nm and 561-nm lasers. Specific gates based on the forward and side scatter were used to identify the bacterial cells (Supplementary Fig. 7). For 10,000 bacterial cells, the emission at 525/40 nm was measured for both excitation at 488 and at 405 nm and the fraction of the emissions for each excitation was calculated. This fraction (488/405 nm excitation) is a proxy for the ATP level in the cell. For each repeat, the fraction of ATP-depleted cells, with a ratiometric ATP measurement below 0.65, was determined. As the ratiometric ATP levels often display a bimodal distribution pattern, this threshold was chosen to be in the middle of the minimum between the two peaks of the distribution.

Measuring membrane depolarization

Membrane potential was measured by diluting the cells 100- to 1000-fold in PBS solution with 1 μ M DIBAC₄(3) (Invitrogen), a fluorescent dye that only stains cells that exhibit membrane depolarization⁵⁸. The fluorescence of 10,000 bacterial cells, gated based on forward and side scatter (Supplementary Fig. 7), was measured at an excitation wavelength of 488 nm and an emission wavelength of 525/40 nm with the CytoFLEX S flow cytometer (Beckman Coulter Life Sciences). The fraction of cells with membrane depolarization was calculated as the fraction of bacterial cells that showed fluorescence.

Measuring translation

Translation was quantified at the single-cell level with the Click-iT[®] HPG Alexa Fluor[®] Protein Synthesis Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, cells were washed twice in PBS and incubated for 30 minutes at 37 °C in M9 medium with 1% casamino acids and with a 1000-fold dilution of the Click-iT[®] Homopropargylglycine reagent. Following incubation, cells were washed once with PBS and subjected to permeabilization and fixation. After permeabilization and fixation, cells were incubated for 30 minutes with the Click-iT[®] reaction cocktail, washed with the Click-iT[®] reaction rinse buffer and dissolved in filtered PBS. The cells were further diluted 100- to 1000-fold in filtered PBS and the fluorescence of 10,000 bacterial cells, gated based on the forward and side scatter (Supplementary Fig. 7), was measured at an excitation wavelength of 488 nm and an emission wavelength of 525/40 nm using the CytoFLEX S flow cytometer (Beckman Coulter Life Sciences). For each repeat, the sum of the translation fluorescence for the 10,000 bacterial cells was calculated. A negative control was also included in which the *E. coli* pBAD33Gm strain that was grown for 2, 4 and 8 hours was treated with 35 μ g/ml chloramphenicol during the incubation in M9 medium with homopropargylglycine to inhibit translation.

Catalase assay

Catalase activity was measured using the catalase assay on a glass microscopy slide. These experiments were performed for cells from an overnight culture and for cells which were grown overnight, diluted in flasks and grown again for 72 hours. Cells were plated on LB plates with 1.5% agar and incubated for 16 hours at 37 °C. This was done for 3 repeats of each strain. For each repeat, 3 colonies were tested. Each colony was streaked on a glass slide and a drop of freshly prepared 3% hydrogen peroxide was added. The amount of bubbles generated was examined and rated.

FRAP microscopy

For the FRAP measurements, a Nikon TiE A1R microscope with cage incubator was used. *E. coli* *ibpA-msfGFP* pBAD33Gm-*obgE* was grown for 8 and 40 hours in flasks. These experiments were only performed in a strain overexpressing *obgE* as only these aggregates were big enough to bleach partially²⁵. The cells were diluted 10-fold in MgSO₄ and spotted on pads of 10 mM MgSO₄ with 2% agarose. The FRAP analysis was performed on 20 and 10 aggregates of cells that were grown for 8 and 40 hours, respectively. A part of the IbpA aggregates was bleached with a 488 nm laser at a power of 100% and a scan speed of 4 frames/second. For the imaging before and after bleaching, the same 488 nm laser was used for excitation, but at a power of 0.5–2% and a scan speed of 1 frame/second, and emission was measured at 500/50 nm. Three images were taken before bleaching at intervals of 4 seconds. The bleaching itself was performed for 4 loops and the recovery after bleaching was monitored every second for a total duration of 50 seconds. For the analysis, the sum of the fluorescence intensity within the bleached area was determined and corrected for fluctuations in fluorescence intensity in the background and in a reference aggregate that remained unbleached. The percentage recovery after bleaching was calculated by dividing the intensity increase after bleaching by the intensity decrease due to the bleaching.

Protein aggregate composition

Aggregate isolation. Aggregate isolation was conducted similar to a previously described protocol⁹¹. For each sample, 50 ml of culture was centrifuged for 30 minutes at 4800 x g and the pellets were frozen at –20 °C until further use. Subsequently, the pellets were washed with 10 ml of buffer A (50 mM HEPES (pH 7.5), 300 mM NaCl, 5 mM β -mercaptoethanol and 1 mM EDTA) and centrifuged for 15 minutes at 4000 x g and 4 °C. Afterwards, the pellet was dissolved in 10 ml of buffer B (buffer A with 1 μ g/ml leupeptin and 0.1 mg/ml 4-(2-aminonoethyl)benzenesulfonyl fluoride hydrochloride). The cells were lysed using the Glen Creston Cell Homogenizer with pressure between 20,000 and 25,000 psi followed by 4 times 30 seconds sonication at 50% amplitude using the Branson Digital sonifier 50/60 Hz. The lysed cells were pelleted by centrifugation for 30 minutes at 11000 x g and 4 °C. The pellet was washed 4 times in 10 ml of buffer D (buffer A with 0.8% (vol/vol) Triton X-100 and 0.1% sodium deoxycholate which are known to solubilize membrane proteins effectively⁹², thereby facilitating their partitioning into the soluble fraction during the purification process). Finally, the pellet was dissolved in 500 μ l of 8 M urea (pH 8).

Coomassie staining. After their isolation, the aggregates were loaded on an SDS gel (Mini-PROTEAN TGX precast protein gels) and stained with Coomassie blue (R250) as described before²⁵. The intensity of the bands was quantified by using Image Lab Software 6.1. The Coomassie intensity for the different strains is represented in Supplementary Fig. 8.

Protein purification for MS analysis. Protein purification was performed as described before⁹¹. In short, dithiothreitol was added to the isolated aggregates at a final concentration of 0.02 M and this mixture

was incubated for 15 minutes at room temperature. Subsequently, 2-iodoacetamide was added to a final concentration of 0.05 M and the solution was incubated for 30 minutes at room temperature in the dark. Following this, ammonium bicarbonate buffer (0.15 M, pH = 8–8.5) with 0.2 µg/ml trypsin was added to the samples reaching a final concentration of 0.11 M and the mixture was incubated for 16 hours at 37 °C for trypsin digestion. After trypsin digestion, the samples were purified on a C₁₈ spin column (Thermo Fisher Scientific). Finally, the samples were diluted in 10 µl with 5% acetonitrile and 0.1% formic acid for injection into the Q Exactive Orbitrap mass spectrometer (Thermo Scientific, USA).

MS analysis. Peptides were loaded onto a 2 cm C₁₈ trap column (ThermoFisher 164946), which was connected in-line to a 15 cm C₁₈ reverse-phase analytical column (Thermo EasySpray ES904). The separation of the peptides was carried out using 100% Buffer A (0.1% formic acid in water) at 750 bar, with the Thermo EasyLC 1200 HPLC system, and the column temperature maintained at 30 °C. MS analysis was performed following a previously established protocol⁹¹ with each condition represented by three biologically independent replicates. Raw data were collected using Xcalibur 3.0.63 software (Thermo Fisher Scientific), and subsequently converted into MGF files using Proteome Discover version 1.4 (Thermo Fisher Scientific).

The converted data were processed using MASCOT version 2.2.06 (Matrix Science) against the Uniprot *E. coli* database⁹³ using previously described parameters⁹¹. MASCOT results were imported to Scaffold version 4 for protein identification (parameters set to retain proteins with 99% confidence and containing at least two identified peptides with confidence level 95%) and label-free quantitation (based on spectrum counting). Finally, protein identities and the total count of unique spectra were used for downstream analyses, including further statistical processing and interpretation.

Analysis of MS data. The proteins present in the aggregates were analyzed in two different ways. To check which proteins were present in the aggregates of the different strains (Fig. 2 and Supplementary Fig. 3), we conducted a qualitative analysis. In this analysis, we tested which types of proteins were enriched in the aggregates compared to the *E. coli* proteome by using a KEGG⁵⁶ and COG⁵⁵ enrichment analysis. To determine which proteins were significantly sequestered in the aggregates over time (Fig. 3), we performed a label-free quantitative analysis based on spectral counting, which is a straightforward and widely used method but comes with certain limitations. These include reduced sensitivity in detecting small differences in protein abundance, and variability between experimental runs. However, in this study, all samples were analyzed in a single run to minimize variability, and comparisons were carefully controlled to minimize these limitations. In this analysis, the quantitative protein abundance data from the MS analysis were compared between different sample groups using the *DESeq2* package in R⁵⁹. To remove proteins with very low abundance, the data underwent pre-filtering, retaining only those proteins with a count higher than 1 in at least 3 samples. Following this pre-filtering step, the *DESeq2* package was used to identify proteins that were significantly enriched or depleted between different sample groups based on a Wald test with false discovery rate (FDR)-controlled multiple testing correction. The significance threshold was set at $P < 0.05$. For each time point, a list with proteins that were significantly enriched in the aggregate was obtained. To see which types of proteins were mainly enriched, this list was also subjected to a KEGG⁵⁶ and COG⁵⁵ enrichment analysis. The *STRINGdb* package in R was used to associate KEGG categories to a list of proteins and to test which categories were enriched⁹⁴. This enrichment was determined by a hypergeometric test. Multiple testing correction was based on the FDR, which was calculated based on the Benjamini-Hochberg procedure. COG categories were assigned to a list of proteins using the

eggNOG tool⁹⁵. In the same manner as before²⁵, enrichment of COG categories was determined using the one-sided Fisher's exact tests with a Bonferroni correction for multiple testing.

AFM-IR spectroscopy

E. coli pBAD33Gm-*obgE* was grown for 2, 8 and 40 hours after dilution and induction. Pellets were harvested, washed twice with 1 ml PBS and fixed in 500 µl of a 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer for 1 hour at room temperature. After washing in 500 µl 0.1 M cacodylate buffer, the samples were fixed in 500 µl of a 1% OsO₄ solution in 0.1 M cacodylate buffer for 2 hours at room temperature. The pellets were washed twice in 0.1 M cacodylate buffer and dehydrated in an ethanol series (30, 50, 70, 90, 100, 100, 100%), rotating slowly for 10 minutes at 4 °C. Subsequently, cells were treated twice with 500 µl propylene oxide, rotating for 15 minutes at 4 °C.

The epoxy embedding was done in three stages, first by resuspending in a 1:1 epoxy and propylene oxide mixture, supplementing 27 µl benzyldimethylamine (BDMA) per 1 ml epoxy, and incubating for 1 hour at 4 °C while rotating. Second, the samples were resuspended in a 2:1 mixture (with BDMA) and left to dry overnight. Finally, the samples were incubated in 100% epoxy resin (with BDMA), drying at low vacuum for 4 hours and polymerizing at 60 °C for 2 days. The resin blocks were sectioned to a thickness of 95 nm and the sections were transferred onto silicon wafers (Ted Pella, 16008), which were then glued to a sample disc (Bruker, SD-102).

For the AFM-IR measurements, a cantilever ($k = 0.2$ N/m and $f_0 = 13$ kHz, PR-EX-nIR2-10, Bruker) was mounted in a nanoIR3 (Bruker) equipped with a MIRcat-QT laser (DRS Daylight Solutions), maximizing the laser sum and optimizing the vertical and lateral deflection to approximately -0.3 V and 0 V respectively. With the laser power set to 1.37%, the pulse length at 160 ns and the pulse rate around 880 kHz, the IR beam was aligned in x and y for each of the QCL chips (at 1730, 1260, 1088, 914 cm⁻¹). The z position was optimized for 1730 cm⁻¹. A phase offset was chosen to maximize the IR amplitude and the phase-locked loop (PLL) gains were set to $I = 0.1$ and $P = 1$. An incident power spectrum was acquired for every sample. Maps were then collected at 1625 cm⁻¹ with a field of view of 20x20 µm, resolution 512x512 px, scan rate 0.1 Hz, AFM gains $I = 2$, $P = 1$ and PLL gains $I = 6$, $P = 60$. Afterwards, the IR focus was re-aligned (only x and y) and a new power spectrum was collected. Spectra were collected on presumptive locations of aggregates, cytoplasm and epoxy matrix, with 5 coaverages and with PLL gains $I = 0.1$, $P = 1$ and spectral resolution of 2 cm⁻¹. To change samples, the sample was moved to its lowest position and replaced with a set of tweezers, taking care not to touch the AFM head. Replicates 2 and 3 were scanned with a different cantilever than replicate 1.

To discriminate between spectra with high and low beta sheet signal and to correlate them with the IR images, we performed k-means clustering on the second derivative of the amide I band (1580 cm⁻¹–1700 cm⁻¹).

Statistics

All measurements were taken from distinct samples. Data were analyzed using GraphPad Prism 9 or R 4.3.0⁹⁶.

A logistic growth model, using the least squares method, was fitted to the data representing the fraction of cells with IbpA and Ph aggregates, with ATP depletion and with membrane depolarization. Outliers and time points where this fraction already started declining were excluded from the analysis to achieve the best possible fit. Based on the fitted logistic growth models, the time required to reach 10 and 90% of the maximal value could be calculated.

Before statistically comparing different groups of data, their normality was assessed with the D'Agostino and Pearson test. To obtain normality, a log transformation was applied to the data describing translation, viable cells and CFUs. Normally distributed data of two or more groups were analyzed with unpaired *t*-tests or two-way

ANOVA, respectively. Non-normally distributed and paired data were analyzed with the Wilcoxon signed-rank test. The appropriate multiple testing corrections were applied when necessary.

A Pearson correlation analysis was conducted to calculate the correlation between the timing of IbpA aggregation, Ph aggregation, persistence and VBNC cell formation. A representation of the raw data and the least square regression line on which the Pearson R values are based is given in Supplementary Fig. 9.

To determine the half-time of recovery after FRAP for each aggregate, a one-phase association model, using least squares regression, was fitted to each repeat of the recovery data.

Data are mainly represented as the mean $\pm$ the standard error of the mean (SEM) and every experiment was measured for at least 3 biological repeats. The levels of significance are: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ***** $P < 0.00001$.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data generated in this study are provided in the Supplementary Information and in the Source Data file which has been deposited, along with MS quantitative counts, in the Zenodo database under accession code [14197261](https://doi.org/10.5281/zenodo.14197261). Mass spectrometry proteomics data have also been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers [PXD059817](https://doi.org/10.26434/chemrxiv-2024-pxd05) and [PXD059868](https://doi.org/10.26434/chemrxiv-2024-pxd05). Source data are provided with this paper.

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Author contributions

Conceptualization and methodology: C.B., L.D., J.M.; Investigation: C.B. was involved in all experiments, S.L., F.D. and D.H. performed microscopy, cytometry and persistence/VBNC assays, W.D. conducted AFM-IR, Ladan K. and Laleh K. performed proteomics; Writing of the original draft: C.B.; Review and editing of draft: C.B., S.L., W.D., Ladan K., Laleh K., J.S., F.R., L.D., J.M.; Figures: C.B.; Supervision: L.D., J.M.

Competing interests

The authors declare no competing interests.

Additional information

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