

37th OTDK Biology Section

# Subcellular localization and rearrangement of bacterial single-stranded DNA binding (SSB) protein condensates in stress response and cell proliferation

*TDK ESSAY*

By:

Júlia Szittyai

Biotechnology MSc

Year 6

Supervisors:

Dr. Péter Ecsédi

Dr. Mihály Kovács

Eötvös Loránd University

Faculty of Science

Institute of Biology

Budapest, 2025



**ELTE**  
EÖTVÖS LORÁND  
TUDOMÁNYEGYETEM

# Table of contents

|                                                                                                               |    |
|---------------------------------------------------------------------------------------------------------------|----|
| Abstract.....                                                                                                 | 2  |
| List of abbreviations .....                                                                                   | 3  |
| List of figures .....                                                                                         | 4  |
| List of tables.....                                                                                           | 4  |
| Introduction.....                                                                                             | 5  |
| Scientific aims .....                                                                                         | 7  |
| Materials and Methods .....                                                                                   | 8  |
| Stressors used in the <i>in vitro</i> and <i>in vivo</i> studies .....                                        | 8  |
| <i>E. coli</i> strain.....                                                                                    | 9  |
| Optimization of fluorescent imaging of EcSSB condensates <i>in vivo</i> .....                                 | 9  |
| Super-resolution microscopy.....                                                                              | 10 |
| Turbidity assays .....                                                                                        | 11 |
| Detecting the condensates of isolated EcSSB in fluorescence microscopy .....                                  | 11 |
| Results.....                                                                                                  | 12 |
| The subcellular localization of EcSSB condensates does change in response to stress .....                     | 12 |
| EcSSB condensates differ during cell growth .....                                                             | 14 |
| EcSSB condensates are more stable in stationary phase.....                                                    | 16 |
| Localization of DNA and EcSSB condensates .....                                                               | 18 |
| Stressors not affecting the EcSSB formed LLPS <i>in vitro</i> indicates indirect effects <i>in vivo</i> ..... | 19 |
| Discussion .....                                                                                              | 20 |
| Acknowledgements .....                                                                                        | 22 |
| References.....                                                                                               | 23 |

# Abstract

The importance of liquid-liquid phase separation (LLPS) is becoming increasingly evident, with numerous eukaryotic and prokaryotic proteins reported to be capable of undergoing LLPS. Single-stranded DNA binding proteins (SSBs) are universal and essential elements of genome maintenance. In 2020, our group demonstrated that the *E. coli* SSB (EcSSB) exhibits LLPS propensity and undergoes phase separation *in vitro* but the behavior of these condensates within bacterial cells remains unexplored. In this study, we aimed to investigate the subcellular localization of SSB condensates under various conditions. To achieve this, we used an *E. coli* strain expressing GFP-fused SSB from a genomic locus. Our results from structured illumination microscopy (SIM) reveal that SSB condensates do rearrange when cells are exposed to stress. The data also indicate that SSB condensate dynamics are linked to metabolic readjustments associated with cell proliferation phases and changes in nutrient conditions. These findings demonstrate that SSB-driven condensates play a role in the storage/sequestration of genome metabolic proteins under normal conditions and can be rapidly mobilized upon stress-induced exposure of genomic ssDNA segments. These results pave the way for genetic and pharmacological engineering of SSB-driven condensation, allowing for the dissection of its cellular roles and the exploration of new opportunities for developing antibacterials targeting this mechanism.

The author of this OTDK thesis carried out the optimization experiments using epifluorescence microscopy for the *in vivo* imaging; performed sample preparation for super-resolution microscopy imaging of cells; conducted the *in vitro* experiments, including turbidity measurements and epifluorescent imaging with isolated EcSSB protein; and participated in the analysis and evaluation of the experimental data.

## List of abbreviations

|                   |                                                |
|-------------------|------------------------------------------------|
| <i>CTP:</i>       | C-terminal peptide                             |
| <i>DMSO:</i>      | dimethyl sulfoxide                             |
| <i>DNA:</i>       | deoxyribonucleic acid                          |
| <i>EcSSB:</i>     | <i>Escherichia coli</i> SSB                    |
| <i>GFP:</i>       | green fluorescent protein                      |
| <i>HU:</i>        | hydroxyurea                                    |
| <i>IDL:</i>       | intrinsically disordered linker                |
| <i>IDR:</i>       | intrinsically disordered region                |
| <i>L-Arg:</i>     | L-arginine                                     |
| <i>LB:</i>        | Luria–Bertani medium                           |
| <i>LLPS:</i>      | liquid-liquid phase separation                 |
| <i>MES:</i>       | 2-(N-morpholino)ethanesulfonic acid            |
| <i>MMS:</i>       | methyl methanesulfonate                        |
| <i>OB domain:</i> | oligonucleotide/oligosaccharide-binding domain |
| <i>SIM:</i>       | structured illumination microscopy             |
| <i>SSB:</i>       | single-stranded DNA binding protein            |
| <i>ssDNA:</i>     | single-stranded DNA                            |
| <i>TIRF:</i>      | total internal reflection fluorescence         |

## List of figures

|                                                                                                                                                                                   |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1: Examples of liquid-liquid phase separated organelles in a eukaryotic cell (A) [11].<br>Structure of <i>E. coli</i> SSB (B) [12] .....                                   | 6  |
| Figure 2: Representative images of cells in the exponential (A), stationary (B) and death (C) phases,<br>evaluated with the script written to identify and analyse droplets ..... | 10 |
| Figure 3: Representative images of cells in the exponential phase evaluated with a script developed<br>for determining signal% in condensates .....                               | 11 |
| Figure 4: The effect of stressors on cells in the exponential phase .....                                                                                                         | 13 |
| Figure 5: The effect of cell growth on unstressed cells .....                                                                                                                     | 15 |
| Figure 6: The effect of stressors on cells in the stationary phase .....                                                                                                          | 17 |
| Figure 7: Cells expressing GFP-EcSSB (green) and stained with Hoechst DNA dye (blue) are<br>shown in the exponential (A, C) and stationary (B, C) growth phases .....             | 19 |
| Figure 8: <i>In vitro</i> turbidity measurements (upper) and epifluorescent microscopy images (below)<br>of EcSSB in the presence of stressors .....                              | 20 |

## List of tables

|                                                                                     |    |
|-------------------------------------------------------------------------------------|----|
| Table 1: Concentrations and effects of stressors used in this study .....           | 8  |
| Table 2: The effect of stressors on cells in the exponential phase in numbers ..... | 14 |
| Table 3: The effect of cell growth on unstressed cells in numbers .....             | 16 |
| Table 4: The effect of stressors on cells in the stationary phase in numbers .....  | 18 |

# Introduction

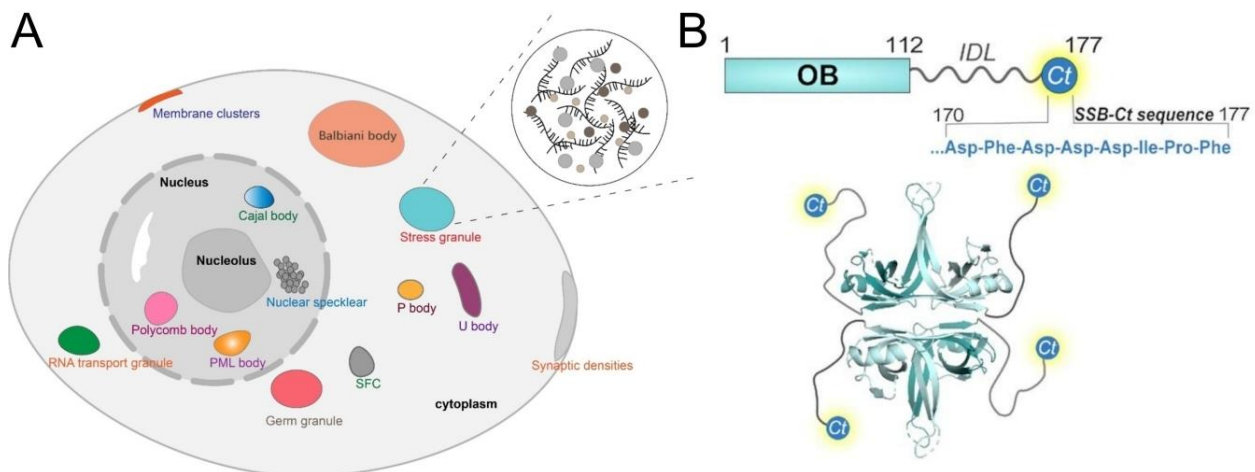
Preserving genome integrity is essential for cell viability, as disruptions in genetic material can threaten survival. Living cells must respond quickly and effectively to DNA damage to handle its potentially serious impacts. Ionizing and ultraviolet radiation, alkylating agents, and polycyclic aromatic hydrocarbons are examples of causes of exogenous DNA damage [1]. DNA, however, is not only susceptible to external damaging factors but is also vulnerable during regular DNA metabolism processes like DNA replication, repair, and recombination. All typical genome maintenance processes and DNA damage usually result in exposed single-stranded DNAs (ssDNAs) as intermediates. Managing these molecules is critical [2] and one protein group responsible for this is single-stranded DNA binding proteins (SSBs).

SSBs are ubiquitous in both eukaryotes and prokaryotes and play an essential role in genome maintenance. As their name indicates, SSBs bind ssDNAs to protect them from chemical degradation and to prevent their reannealing. In addition to their protective function, SSBs have an organizational role in DNA metabolism, recruiting and mobilizing other genome maintenance proteins through protein–protein interactions at sites of action. Members of the SSB interactome can be grouped by their role in DNA metabolism and can be categorized as proteins that are involved in replication, recombination, replication restart, repair and other processes [2]. One of the most studied and known SSBs in prokaryotes is the *Escherichia coli* SSB (EcSSB).

EcSSB is a 178-amino acid protein of about 19 kDa that forms homotetramers. It can be divided into two main regions: the oligonucleotide/oligosaccharide-binding (OB) domain and the intrinsically disordered linker/region (IDL/IDR). The last nine amino acids of the latter form a highly conserved region usually termed the C-terminal peptide (CTP). The function of the OB domain is to bind ssDNA through protein-nucleic acid interactions, while the IDL and mostly the CTP is responsible for partner binding [3]. Members of this expansive protein-protein interaction network include for example RecQ helicase [4], uracil-DNA glycosylase [5] and RecA protein [6]. Experimental data show that mutations of the SSB protein cause disruptions in the processes involving its interacting partners, and the emergence of temperature or UV sensitive, or even non-viable phenotypes [7]. The IDL has no conserved amino acid sequence in bacteria, except for the CTP, however, this region mediates its liquid-liquid phase separation (LLPS) propensity which was

first studied in detail by our group in 2020 [3]. Our results also showed that an increased concentration of ssDNA has an inhibitory effect on phase separation and dissolves EcSSB condensates, suggesting that this phenomenon could be related to the genome maintenance function of EcSSB.

LLPS has been known for nearly a century, yet its importance has only been recognized in recent years. Numerous proteins were reported to be capable of phase separation [8] and LLPS was shown to be a key mechanism for the formation of membraneless organelles. Examples of these structures include Cajal bodies and P granules [9]. The formation of these liquid-like particles is typically driven by weak multivalent protein-protein or protein-nucleic acid interactions. They are dynamic structures distinct from the cytoplasm but with fluid-like properties. Since they are not bound by membrane, molecules can easily enter and exit the condensates, which can easily increase or decrease in size or even disappear. These structures can serve a wide range of functions, such as storage, rapid mobilization of biomolecules, increasing the local concentration of specific molecules within the condensate, and enriching interacting partners involved in biochemical processes, thereby enhancing reaction rates [10]. Despite the growing data about LLPS in eukaryotes, the field of LLPS, especially in bacteria, remains largely unexplored. However, in recent years, it has become increasingly apparent that this phenomenon is equally important in their case.



**Figure 1: Examples of liquid-liquid phase separated organelles in a eukaryotic cell (A) [11]. Structure of *E. coli* SSB (B) [12]**

Prokaryotic cells lack internal membrane systems, thus these membraneless organelles could be of key importance for subcellular organization and the regulation of cellular processes, potentially

providing advantages for the cells, such as increased stress tolerance. One of the main obstacles in studying bacterial condensates is the difficulty of visualization at this scale. Bacteria are at least an order of magnitude smaller than eukaryotic cells, requiring the use of super-resolution microscopy for *in vivo* analysis [10], thus the identification of proteins and complexes prone to LLPS is often conducted through *in vitro* experiments. Despite these challenges, several membraneless organelles have already been identified in bacteria [13], [14], [15]. In 2019 Zhao *et al.* used super-resolution microscopy to investigate the intracellular localization of EcSSB in an *E. coli* strain expressing a GFP-fused EcSSB variant from a genomic locus. In an unstressed state, EcSSB was found to localize mainly near the membrane in many small foci and, to a lesser extent, at replication sites. Upon mitomycin C treatment, the resulting DNA damage resulted in EcSSB dissociating from the membrane with nearly all SSB being found inside the cell. [16]. Estimates suggest that by default, only a small fraction of the SSB in the cell at any given time is associated with the genome, e.g. at replication forks, the rest appears to be stored [17]. The biological significance of this large surplus is not yet fully understood.

## Scientific aims

Our previous findings showed that EcSSB undergoes LLPS *in vitro* under physiologically relevant conditions but the dynamic localization of protein condensates *in vivo* has not been studied in detail. Therefore our aims were:

- To investigate the dynamics of the subcellular localization of phase-separated EcSSB condensates during stages of cell growth and in the presence of stressors (both physiological and genomic).
- To confirm the *in vitro* results and *in vivo* suggestions of Harami *et al.* [3] by studying the relationship between genome metabolism, the emergence of ssDNA and the state of the condensates.
- To examine the effects of the stressors on protein condensates *in vitro* to determine whether the observed effects in the bacterial cells are the result of direct phase separation inhibition or their impact on cellular processes.

# Materials and Methods

## Stressors used in the *in vitro* and *in vivo* studies

Materials used in this study, their source, catalog number, effects, as well as their concentration or dose used in the *in vitro* and *in vivo* microscopy experiments are summarized in Table 1.

*Table 1: Concentrations and effects of stressors used in this study*

| Stressor<br>(source)                                        | <i>In vitro</i> dose   | <i>In vivo</i> dose                    | Effects                                                                             |
|-------------------------------------------------------------|------------------------|----------------------------------------|-------------------------------------------------------------------------------------|
| UV<br>(UVP CX-2000 UV Crosslinker)                          | 600 mJ/cm <sup>2</sup> | 600 mJ/cm <sup>2</sup>                 | DNA damage: covalently binds pyrimidine bases, initiates SOS response [18], [19]    |
| L-Arginine<br>(Sigma-Aldrich, A8094-25G)                    | 500 mM                 | 100 mM                                 | Supports bacterial growth (metabolism)                                              |
| Hydroxyurea<br>(Sigma-Aldrich, H8627-10G)                   | 500 mM                 | 100 mM                                 | Inhibits ribonucleotide reductase and thus DNA synthesis reversibly [20]            |
| Diamide<br>(Sigma-Aldrich, D3648-5G)                        | 5 mM                   | 1 mM                                   | Specific thiol oxidizing agent, causes Cys oxidation [21]                           |
| Glucose<br>(Sigma-Aldrich, G8270)                           | 2%                     | 0.5%                                   | Supports bacterial growth (metabolism)                                              |
| H <sub>2</sub> O <sub>2</sub><br>(VWR Chemicals, 23619.297) | 5 mM                   | 1 mM                                   | Oxidizing agent, DNA damage, protein denaturation [22]                              |
| NaCl<br>(VWR Chemicals, 27800.291)                          | 1 M                    | 200 mM                                 | Physiological (osmotic) stress; inhibits EcSSB phase separation <i>in vitro</i> [3] |
| MES (pH 6.0)<br>(Sigma-Aldrich, M2933-500G)                 | 100 mM                 | 20 mM                                  | Physiological (pH) stress                                                           |
| MMS<br>(Sigma-Aldrich, 129925-5G)                           | 0.05%                  | 0.01%                                  | Alkylating agent, induces DNA (replication, transcription) damage [23]              |
| Lactose<br>(Molar Chemicals Ltd.,<br>02150-203-190)         | 2%                     | 0.5% (exponential),<br>4% (stationary) | Supports bacterial growth (metabolism)                                              |

|                                              |               |              |                                                                                           |
|----------------------------------------------|---------------|--------------|-------------------------------------------------------------------------------------------|
| Myricetin<br>(Sigma-Aldrich, 72576-10MG)     | 5 $\mu$ M     | 1 $\mu$ M    | Antimicrobial flavonoid [24];<br>inhibits ssDNA binding activity of<br>bacterial SSB [25] |
| Taxifolin<br>(Merck, 78666-25MG-F)           | 50 $\mu$ M    | 10 $\mu$ M   | Antimicrobial flavonoid [24];<br>inhibits ssDNA binding activity of<br>bacterial SSB [26] |
| Nitrofurantoin<br>(Sigma-Aldrich, N7878-10G) | 50 $\mu$ g/mL | 3 $\mu$ g/mL | DNA damage: forms interstrand<br>crosslinks [19], [27]                                    |
| FM 4-64 dye<br>(Invitrogen, T13320)          | 20 $\mu$ M    | 8 $\mu$ M    | Inner membrane dye [16]                                                                   |

### ***E. coli* strain**

For our research we used an *E. coli* strain (#29003) expressing a GFP-fused EcSSB from a genomic locus, which was a kind gift from Piero R. Bianco [16]. Note that GFP-EcSSB can enrich in the condensates formed by the wild type protein, enabling its detection in fluorescent microscope [3].

### **Optimization of fluorescent imaging of EcSSB condensates *in vivo***

Since super-resolution microscopes are not available in house, first the experiments were optimized using an epifluorescent microscope (Nikon Eclipse Ti-E TIRF microscope) in house before transferring the optimized setups to the SIM microscopes in Pécs. Cells were grown overnight in Luria–Bertani medium (LB) containing kanamycin (25  $\mu$ g/ml) and chloramphenicol (34  $\mu$ g/ml). Overnight cultures were diluted 1:100 in fresh LB media containing antibiotics and grown at room temperature at 200 rpm shaking until they reached a certain OD<sub>600</sub> value indicating the different growth phases (exponential or stationary phase). Note that room temperature was used to aid the maturation of GFP thus enhancing the signal during imaging. Cells were centrifuged, washed with 10 mM MgSO<sub>4</sub> twice, then resuspended in MgSO<sub>4</sub> containing the stressors at concentrations indicated in Table 1. The pH of stressor solutions was adjusted to 7.0 with the exception of MES (pH 6.0). Cells were shaken for 30 minutes at 200 rpm at 37 °C and imaged. UV stressed cells were observed without incubation to avoid regeneration. Depending on cell density dilutions were applied before imaging to avoid a disruptive number of cells during imaging. During measurements VWR microscope slides (CAT: 631-1550) with Knittel Glass 20x20 mm coverslips were used.

## Super-resolution microscopy

Super-resolution imaging was carried out similarly to epifluorescence microscopy but here Zeiss High Performance  $0.17 \pm 0.005$  mm thick glass coverslips and a Zeiss Elyra S1 SIM microscope with 488 nm (Lasos) laser were used. Moreover, Hoechst DNA stain images were captured with a Nikon X-Light V3 Deep SIM microscope. Cells were treated with Hoechst for one minute followed by two washing steps before observation.

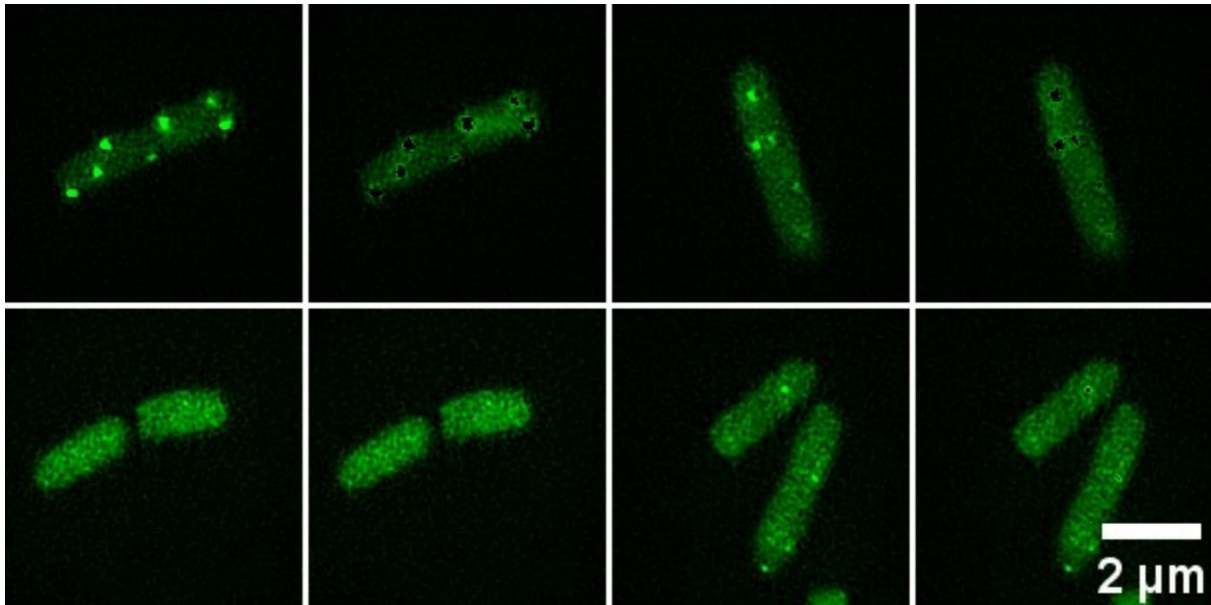
Image analysis was done with Fiji software. For image evaluation purposes, two scripts were developed for automatic quantification of protein condensates in cells and measuring their intensities. Note that this method could also be useful for other groups in the future researching phase-separated droplets *in vivo*. During evaluation the cells were identified by selecting objects above a certain threshold (Li cross-entropy thresholding). This was followed by another thresholding step within the cells to identify spots (condensates) with a modified Find Maxima function (Figure 2).



**Figure 2: Representative images of cells in the exponential (A), stationary (B) and death (C) phases, evaluated with the script written to identify and analyse droplets**

To determine the percentage of fluorescent signal present in the condensates compared to the cytoplasm, another script was developed which outlined the condensates and subtracted them from the original image. The difference between the intensities was expressed as percentage in the

condensates (Figure 3). Colocalization analysis of images of Hoechst-stained cells was performed with Fiji plugin Coloc2.



*Figure 3: Representative images of cells in the exponential phase evaluated with a script developed for determining signal% in condensates*

### **Turbidity assays**

Turbidity measurements were performed at 600 nm, using Thermo Fisher Nunc 384-well, non-treated flat bottom microplates and a Tecan Infinite F Nano+ plate reader instrument. The sample volume was 100  $\mu$ L in a buffer containing 20 mM Tris-OAc pH 7.5, 5 mM MgOAc, 50 mM NaGlu. The EcSSB tetramer concentration was 5  $\mu$ M and 3 technical replicates were measured at room temperature.

### **Detecting the condensates of isolated EcSSB in fluorescence microscopy**

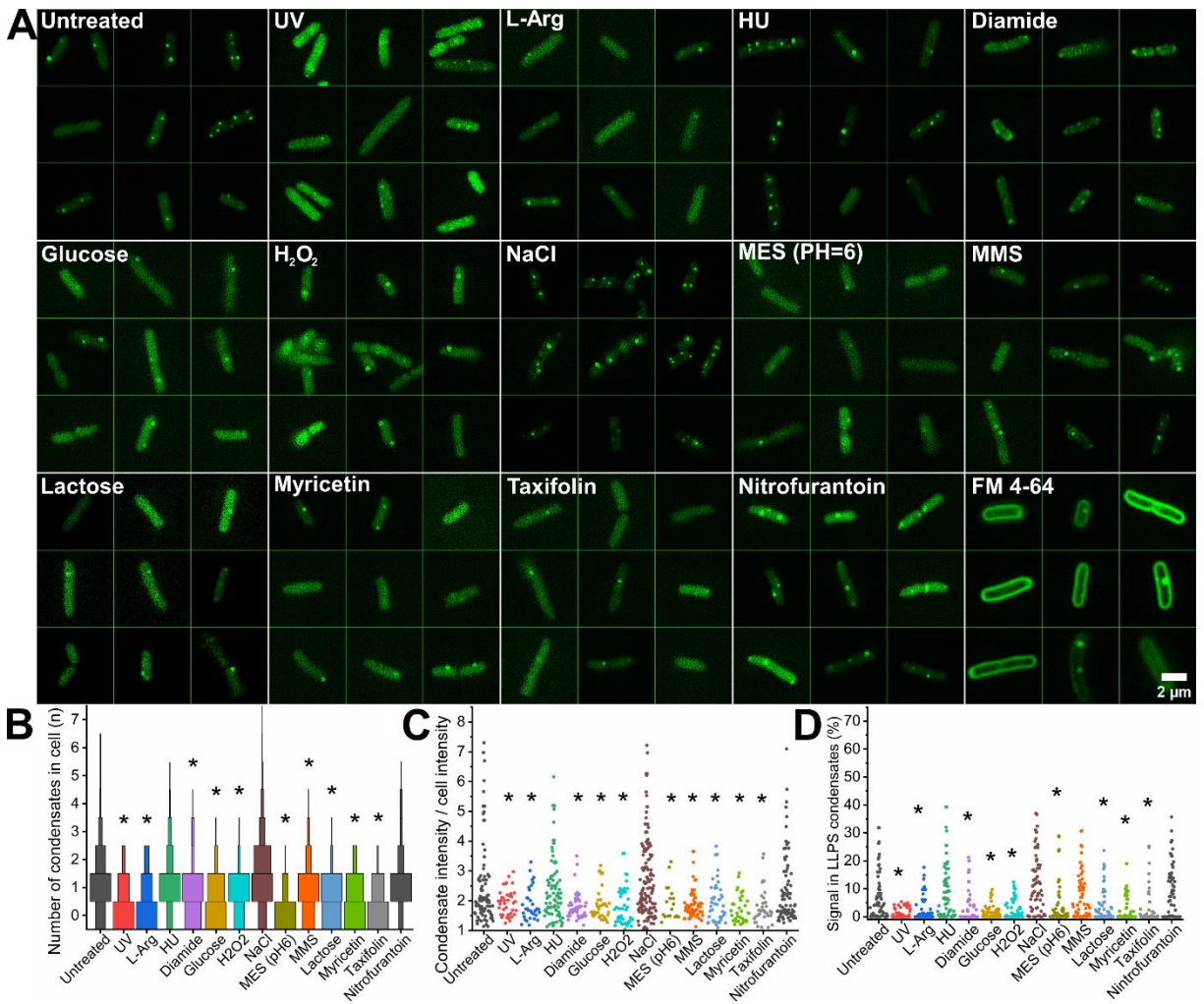
The effects of stressors on EcSSB condensates *in vitro* were also visualized using epifluorescence microscopy. Samples contained 5  $\mu$ M EcSSB tetramers and 0.1  $\mu$ M fluorescein labelled EcSSB which could enrich in the formed protein droplets, thus they could be imaged [3]. Samples were measured on an Ibidi 15 Well  $\mu$ -Slide Angiogenesis slide at room temperature. A Nikon Eclipse Ti-E TIRF microscope was used in epifluorescent mode with a Cyan 488-nm laser (Coherent).

# Results

## The subcellular localization of EcSSB condensates does change in response to stress

Analyzing untreated *E. coli* cells in exponential phase ( $OD_{600} = 0.6$ ), usually one or few (up to 6) small condensates were found within the cytoplasm (Figure 4 and Table 2). Note that a weaker, diffuse GFP signal indicates proteins present outside of protein droplets. Based on GFP signal intensity analyses of cells both with and without protein droplets (Figure 4, D) we found that cells usually store around 10-20% of their total EcSSB in condensates. The signal observed in the droplets does not surpass 40% of the total fluorescence signal in any cell, meaning much of the EcSSB pool is present in diffuse form in the cytoplasm. Strikingly, most of the stressors which were added to the cells caused significantly different distribution of EcSSB condensates (Figure 4). In most cases they were disrupted, resulting either in smaller, less intense protein droplets or their complete dissolution. Here the effects of UV, L-arginine, glucose, MES (pH 6), myricetin and taxifolin were found to be the most significant. Furthermore, several compounds like HU, MMS, nitrofurantoin and NaCl caused mild or no inhibitory effect on EcSSB condensates, the latter even enhanced droplet formation.

At the start of the optimization experiments, the red membrane dye FM 4-64 was used to visualize the bacterial cell membrane [16]. However, it soon became evident that the dye caused artefacts and associated the EcSSB condensates to the membrane (Figure 4, A). Note that Zhao *et al.* [16] used the same membrane dye, which might explain why they observed enriched GFP-EcSSB signal close to the membrane. Following this discovery, we opted to abandon the use of the dye to eliminate its influence when studying the effects of the other stressors. Instead, we included the membrane dye in the stress panel. These images were not evaluated because of technical challenges.



**Figure 4: The effect of stressors on cells in the exponential phase**

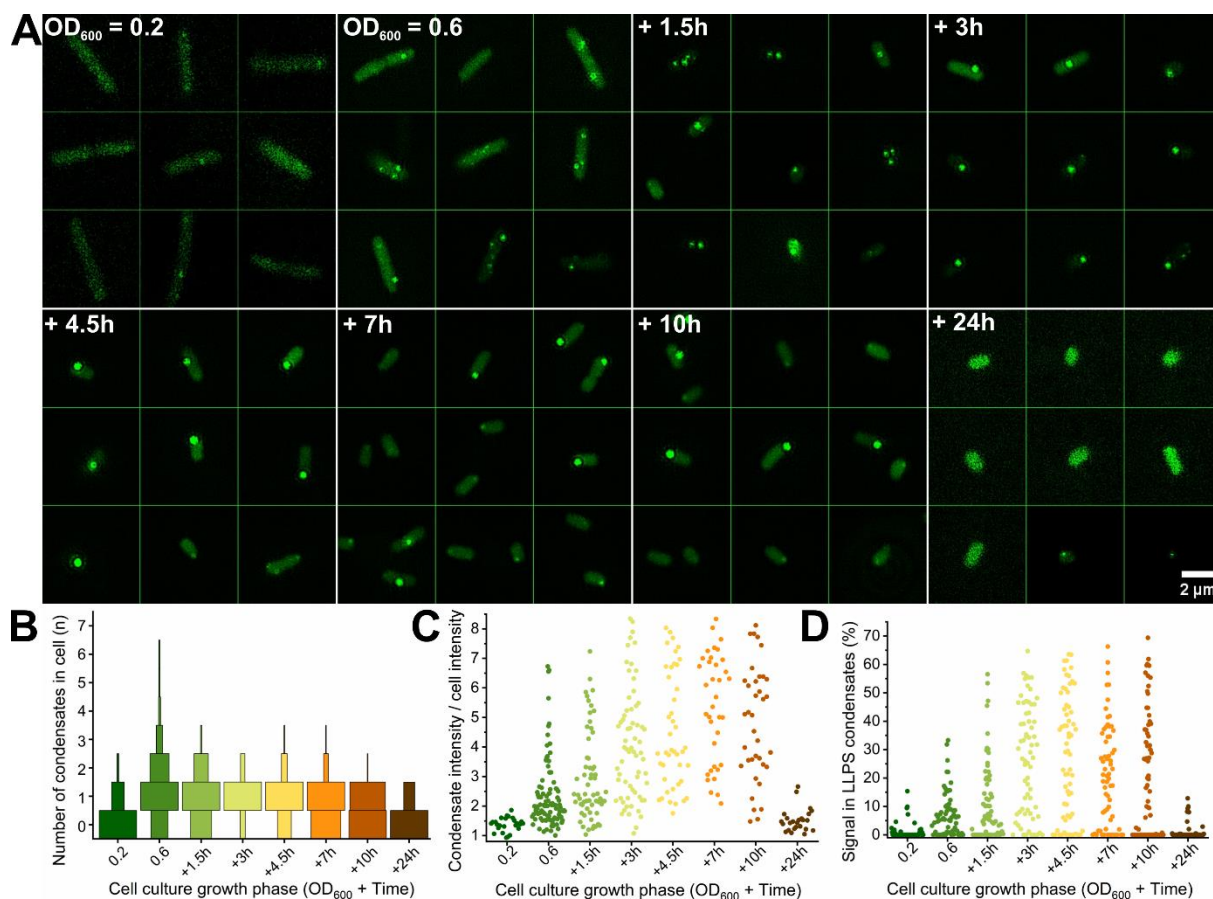
**Table 2: The effect of stressors on cells in the exponential phase in numbers**

|                               | Average number of condensates in cell | Median values | Average I/ $\sigma$ (condensate intensity / cell intensity) | Total number of condensates | Mean signal in LLPS condensates (%) |
|-------------------------------|---------------------------------------|---------------|-------------------------------------------------------------|-----------------------------|-------------------------------------|
| Untreated                     | 1.35 $\pm$ 1.2                        | 1             | 2.55 $\pm$ 1.48                                             | 88                          | 7.17 $\pm$ 8.54                     |
| UV                            | 0.58 $\pm$ 0.63 *                     | 0             | 1.94 $\pm$ 0.43 *                                           | 38                          | 1.03 $\pm$ 1.65 *                   |
| L-Arg                         | 0.51 $\pm$ 0.70 *                     | 0             | 1.88 $\pm$ 0.54 *                                           | 33                          | 2.58 $\pm$ 4.34 *                   |
| HU                            | 1.32 $\pm$ 0.96                       | 1             | 2.40 $\pm$ 1.08                                             | 86                          | 9.09 $\pm$ 10.10                    |
| Diamide                       | 0.75 $\pm$ 0.82 *                     | 1             | 1.83 $\pm$ 0.44 *                                           | 49                          | 3.18 $\pm$ 5.79 *                   |
| Glucose                       | 0.53 $\pm$ 0.65 *                     | 0             | 1.82 $\pm$ 0.54 *                                           | 38                          | 1.57 $\pm$ 2.36 *                   |
| H <sub>2</sub> O <sub>2</sub> | 0.8 $\pm$ 0.61 *                      | 1             | 1.86 $\pm$ 0.65 *                                           | 52                          | 2.38 $\pm$ 3.35 *                   |
| NaCl                          | 1.92 $\pm$ 1.16                       | 1             | 2.73 $\pm$ 1.28                                             | 126                         | 12.00 $\pm$ 10.22                   |
| MES (pH 6)                    | 0.29 $\pm$ 0.46 *                     | 0             | 1.88 $\pm$ 0.61 *                                           | 19                          | 3.62 $\pm$ 6.89 *                   |
| MMS                           | 1.31 $\pm$ 0.81 *                     | 1             | 1.88 $\pm$ 0.49 *                                           | 67                          | 8.64 $\pm$ 8.12                     |
| Lactose                       | 0.68 $\pm$ 0.65 *                     | 1             | 1.94 $\pm$ 0.69 *                                           | 44                          | 3.24 $\pm$ 5.51 *                   |
| Myricetin                     | 0.52 $\pm$ 0.64 *                     | 0             | 1.73 $\pm$ 0.46 *                                           | 34                          | 2.20 $\pm$ 4.01 *                   |
| Taxifolin                     | 0.58 $\pm$ 0.53 *                     | 0             | 1.74 $\pm$ 0.59 *                                           | 38                          | 2.86 $\pm$ 6.08 *                   |
| Nitrofurantoin                | 1.34 $\pm$ 1.01                       | 1             | 2.43 $\pm$ 1.23 *                                           | 79                          | 8.99 $\pm$ 9.33                     |

Values are from 65 cells each (N=65). Means  $\pm$  SDs are shown. Stars indicate significant differences compared to untreated cells.

### EcSSB condensates differ during cell growth

Previous data was obtained by analyzing cells in the exponential phase, however, condensates as storages might function differently in stationary or late growth phases. Thus, after exploring the effects of stressors and stress in exponential phase, unstressed cells were studied at different stages of growth (**Error! Reference source not found.** and Table 3). In the early exponential phase ( $OD_{600} = 0.2$ ), cells exhibit very weak signals (this might be because the GFP-EcSSB production is not yet as intensive as in latter phases and the GFP maturation level is low) and only a few weak protein droplets can be observed. In later exponential and early stationary phases ( $OD_{600} = 0.6 + 1.5h, +3h$  and  $+4.5h$ ) the number of condensates in cells starts to decrease and the majority of the EcSSB pool assembles into one intense focus. Moreover, the GFP signal in the cytoplasm decreases simultaneously. In later phases ( $OD_{600} = 0.6 + 7h, +10h$  and  $+24h$ ) condensates start to disappear and only weak, diffuse GFP signal can be detected in the cytoplasm 24 hours after exponential phase.



**Figure 5: The effect of cell growth on unstressed cells**

Since EcSSB condensates behave differently in the stages of cell growth, it is possible that these changes are caused by the different processes (e.g. DNA replication) characteristic of each stage and imply the role of EcSSB in each phase.

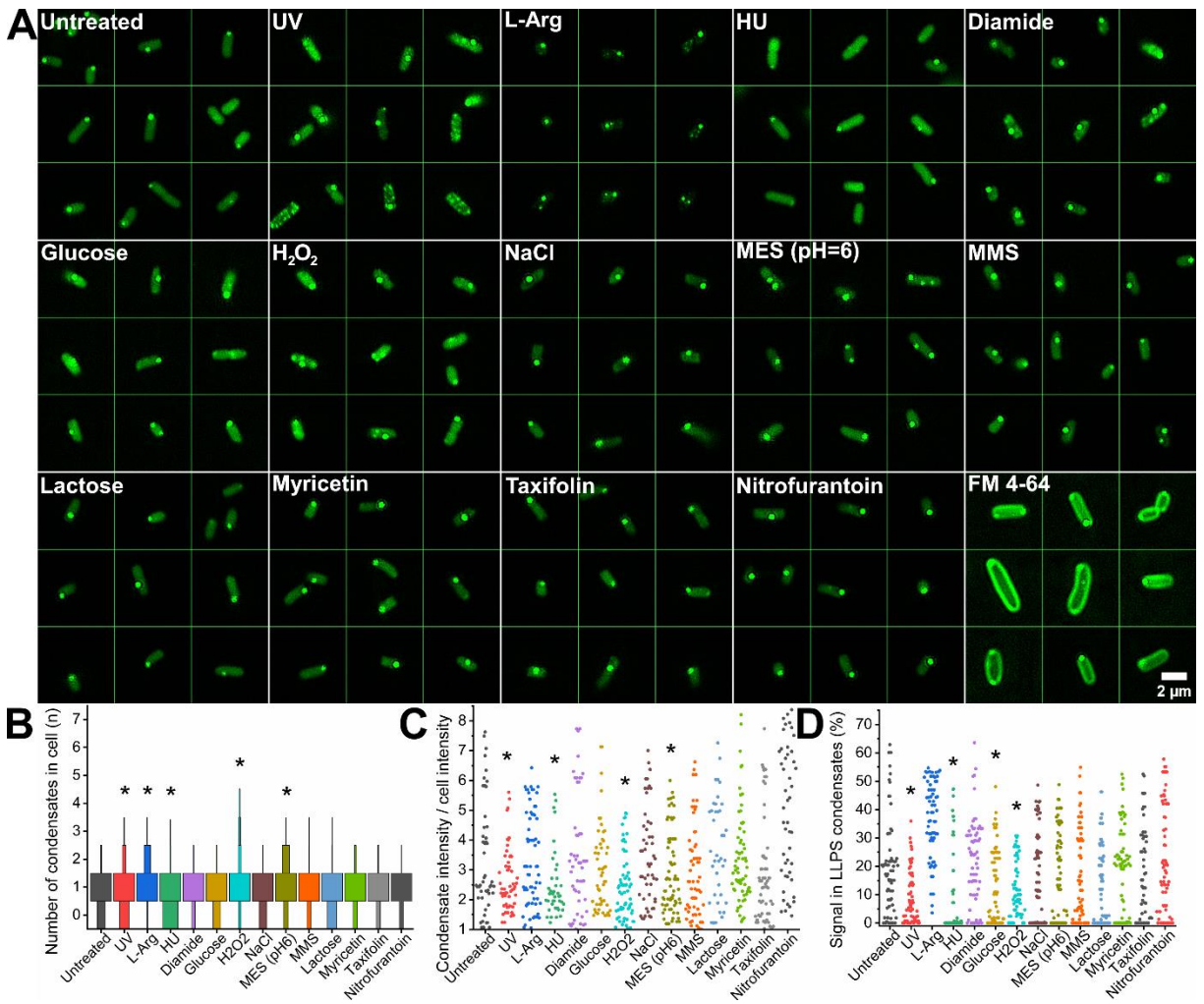
Considering these results, we decided to explore the effects of the stressors in stationary phase cells as well. The  $OD_{600}=0.6$  +3h state was chosen for this experiment because it seemed likely that intensive replication ends here, but the cells would not be completely inactive, which might be true for later stages.

**Table 3: The effect of cell growth on unstressed cells in numbers**

| OD <sub>600</sub> + Time                                     | Average number of condensates in cell | Median values | Average I/σ (condensate intensity / cell intensity) | Total number of condensates | Mean signal in LLPS condensates (%) |
|--------------------------------------------------------------|---------------------------------------|---------------|-----------------------------------------------------|-----------------------------|-------------------------------------|
| 0.2                                                          | 0.30 ± 0.53                           | 0             | 1.37 ± 0.23                                         | 25                          | 1.02 ± 2.72                         |
| 0.6                                                          | 1.37 ± 1.19                           | 1             | 2.34 ± 1.17                                         | 95                          | 7.41 ± 7.76                         |
| +1.5h                                                        | 0.94 ± 0.73                           | 1             | 2.95 ± 1.50                                         | 62                          | 11.55 ± 13.96                       |
| +3h                                                          | 0.98 ± 0.44                           | 1             | 4.09 ± 1.88                                         | 66                          | 26.55 ± 19.06                       |
| +4.5h                                                        | 0.88 ± 0.62                           | 1             | 5.85 ± 3.02                                         | 57                          | 27.69 ± 21.31                       |
| +7h                                                          | 0.67 ± 0.63                           | 1             | 6.05 ± 2.33                                         | 53                          | 21.31 ± 17.82                       |
| +10h                                                         | 0.50 ± 0.52                           | 0             | 6.1 ± 2.81                                          | 50                          | 21.46 ± 21.43                       |
| +24h                                                         | 0.22 ± 0.42                           | 0             | 1.51 ± 0.39                                         | 25                          | 1.12 ± 2.95                         |
| Values are from 65 cells each (N=65). Means ± SDs are shown. |                                       |               |                                                     |                             |                                     |

### **EcSSB condensates are more stable in stationary phase**

Using the same stressors, EcSSB condensates of cells in stationary phase were also analyzed (Figure 6 and Table 4). We found that most stressors do not cause significant changes in the localization of EcSSB, in contrast to the effects experienced in the earlier growth phase. Some of the main disruptors, namely UV, glucose and MES (pH 6) remained the most effective in dissolving condensates, however, the complete dissociation of droplets was only detectable in a few cases and predominantly fragmentation of condensates was observed instead. H<sub>2</sub>O<sub>2</sub> showed a larger effect compared to the exponential phase. Unlike cells in the exponential phase, here cells hold up to 70% of their fluorescent signal in their condensate(s) (Table 4). The results suggest that stationary phase cells, which are in a kind of “resting” state, are less sensitive to stress as far as their EcSSB LLPS is concerned. In this cell growth phase, rapid DNA replication is no longer required, which impacts the dynamics of EcSSB in the cell.



**Figure 6: The effect of stressors on cells in the stationary phase**

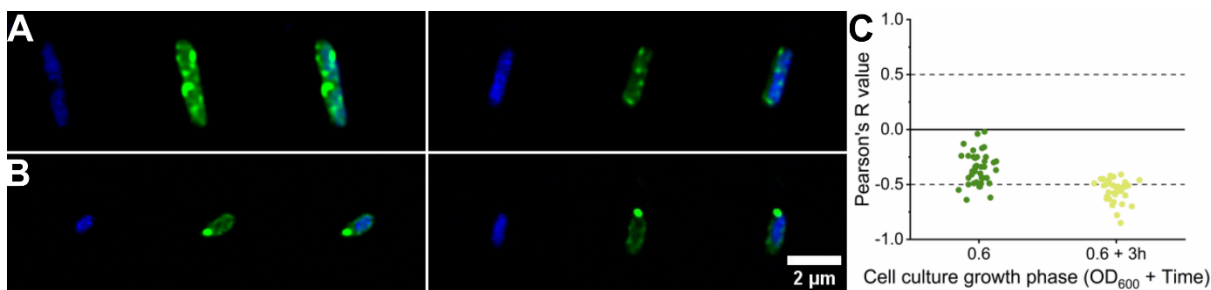
**Table 4: The effect of stressors on cells in the stationary phase in numbers**

|                               | Average number of condensates in cell | Median values | Average I/ $\sigma$ (condensate intensity / cell intensity) | Total number of condensates | Mean signal in LLPS condensates (%) |
|-------------------------------|---------------------------------------|---------------|-------------------------------------------------------------|-----------------------------|-------------------------------------|
| Untreated                     | 0.78 $\pm$ 0.49                       | 1             | 3.36 $\pm$ 1.91                                             | 51                          | 20.08 $\pm$ 16.60                   |
| UV                            | 0.83 $\pm$ 0.62 *                     | 1             | 2.62 $\pm$ 1.06 *                                           | 56                          | 9.12 $\pm$ 9.29 *                   |
| L-Arg                         | 1.20 $\pm$ 0.56 *                     | 1             | 3.18 $\pm$ 1.63                                             | 73                          | 36.46 $\pm$ 13.74                   |
| HU                            | 0.62 $\pm$ 0.57 *                     | 1             | 2.47 $\pm$ 1.10 *                                           | 40                          | 7.81 $\pm$ 14.15 *                  |
| Diamide                       | 0.91 $\pm$ 0.38                       | 1             | 3.17 $\pm$ 2.02                                             | 59                          | 22.04 $\pm$ 14.90                   |
| Glucose                       | 0.76 $\pm$ 0.47                       | 1             | 3.04 $\pm$ 1.44                                             | 51                          | 14.10 $\pm$ 13.05 *                 |
| H <sub>2</sub> O <sub>2</sub> | 0.98 $\pm$ 0.38 *                     | 1             | 2.43 $\pm$ 1.09 *                                           | 63                          | 11.60 $\pm$ 9.86 *                  |
| NaCl                          | 0.74 $\pm$ 0.46                       | 1             | 3.57 $\pm$ 1.74                                             | 53                          | 17.49 $\pm$ 15.86                   |
| MES (pH 6)                    | 1.10 $\pm$ 0.62 *                     | 1             | 2.85 $\pm$ 1.37 *                                           | 74                          | 19.08 $\pm$ 15.74                   |
| MMS                           | 0.87 $\pm$ 0.45                       | 1             | 2.95 $\pm$ 1.55                                             | 59                          | 17.25 $\pm$ 16.10                   |
| Lactose                       | 0.73 $\pm$ 0.53                       | 1             | 3.72 $\pm$ 1.68                                             | 39                          | 13.01 $\pm$ 13.69                   |
| Myricetin                     | 0.90 $\pm$ 0.45                       | 1             | 3.41 $\pm$ 1.56                                             | 64                          | 19.96 $\pm$ 15.07                   |
| Taxifolin                     | 0.88 $\pm$ 0.42                       | 1             | 3.24 $\pm$ 2.35                                             | 61                          | 18.44 $\pm$ 16.28                   |
| Nitrofurantoin                | 0.85 $\pm$ 0.39                       | 1             | 5.67 $\pm$ 2.77 *                                           | 57                          | 22.79 $\pm$ 19.18                   |

Values are from 65 cells each (N=65). Means  $\pm$  SDs are shown. Stars indicate significant differences compared to untreated cells.

### Localization of DNA and EcSSB condensates

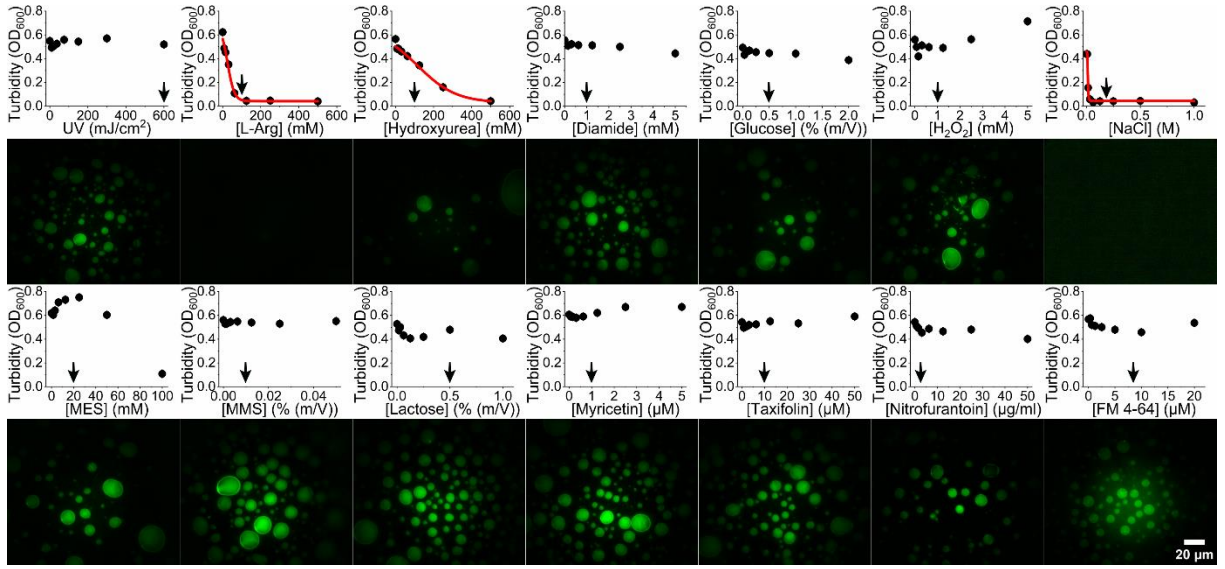
Our previous data show that ssDNA has an inhibitory effect on EcSSB droplets [3]. This data suggests that prokaryotic DNA and EcSSB condensates do not colocalize in the cytoplasm. To examine the correlation between DNA and EcSSB assemblies, we used Hoechst DNA dye and analyzed the cells in the exponential and stationary growth stages (Figure 7). It appears that the GFP (in the condensates) and the Hoechst signal separate in space in both phases, but more apparently in the stationary phase. These results support that EcSSB and DNA (including ssDNA) exist separately in the cytoplasm (with EcSSB accumulating into condensates) when the cells are in a resting state and DNA work is significantly reduced. It is also important to note that the colocalization in exponential phase cells can be largely attributed to the disperse GFP-EcSSB signal instead of the condensates, while in stationary phase, where most of the EcSSB is in condensates, there is little to no colocalization.



**Figure 7: Cells expressing GFP-EcSSB (green) and stained with Hoechst DNA dye (blue) are shown in the exponential (A, C) and stationary (B, C) growth phases**

### **Stressors not affecting the EcSSB formed LLPS *in vitro* indicates indirect effects *in vivo***

To follow the formation of protein condensates and the effects of stressors *in vitro*, turbidity assays ( $\lambda=600$  nm) were carried out together with epifluorescent imaging (Figure 8). Note that the black arrows indicate the concentrations of stressors used during *in vivo* experiments and dose-response curve was fitted to data points in cases when the stressors inhibited EcSSB condensates. We found that most stressors do not affect the formation of EcSSB condensates *in vitro* even at large concentrations meaning the effects observed *in vivo* are caused by the stressors affecting protein droplets via cellular processes. Here NaCl, L-Arg and HU were exceptions as shown in Figure 8. Note that the *in vitro* inhibitory effect of NaCl was previously found by Harami *et al.* [3]. Imaging showed spherical phase separated droplets indicating that the signal in turbidity was not caused by protein aggregation. In the case of NaCl, L-Arg and HU few or no condensates were present in epifluorescent microscope. A slight increase in turbidity was observed in the case of H<sub>2</sub>O<sub>2</sub>, and irregularly shaped particles were present in microscope images, both of which could indicate slight protein aggregation. Another interesting stressor is MES (pH 6). As its concentration increases, first, a slight increase then a decrease can be detected in turbidity values. A possible explanation for this could be that at lower concentrations MES as a buffer component or the lower pH (6 instead of 7) supports phase separation by improving protein stability or enhancing interactions, while at higher concentrations it exhibits an inhibitory effect.



**Figure 8: In vitro turbidity measurements (upper) and epifluorescent microscopy images (below) of EcSSB in the presence of stressors**

## Discussion

Our group previously showed that EcSSB is capable of LLPS *in vitro* [3], however, the significance of this phenomenon in living cells as well as the localization and regulation of these protein condensates *in vivo* is not yet explored. Zhao *et al.* [16] were the first and the only ones yet to follow EcSSB droplets *in vivo* using super-resolution imaging, however they did not analyze the behavior of these particles in detail.

In this study we investigated the subcellular localization of EcSSB condensates and found with the use of super-resolution microscopy that they can be influenced by various conditions. Phase-separated EcSSB condensates dissolve or fragmentate under numerous stress conditions and they also behave differently in various growth phases of bacteria, most likely in line with the distinct genome metabolism processes occurring in each stage. We also determined that while some stressors still exhibited their effects on stationary phase cells, these cells, or more precisely their EcSSB droplets were a lot less responsive to exogenous impacts, suggesting that LLPS in replicating cells, in which intensive DNA replication is ongoing, is more dynamic.

Moreover, data shows that DNA and EcSSB (in the form of condensates) practically do not colocalize in cells. This finding provides *in vivo* proofs to our previous suggestion that the LLPS of EcSSB plays a role in the storage/sequestration of genome metabolic proteins under normal

conditions and can be rapidly mobilized upon stress-induced exposure of genomic ssDNA segments [3]. The likely function of storing EcSSB separately from the cytoplasm might be advantageous in the long term by preventing EcSSB to interact with the genome nonspecifically thus maintaining genome integrity. At the same time, a storing mechanism like this ensures the availability of EcSSB in large quantities when it is quickly needed, allowing for a rapid response to DNA damage. It is worth noting here that the developed image evaluation methods and the connected automatized Fiji scripts written for this study can be of interest for a wider audience working with LLPS forming proteins.

*In vitro* turbidity measurements and epifluorescent microscopy imaging revealed that with few exceptions, the stressors used *in vivo* did not inhibit LLPS directly. This indicates that the effects of the stressors on living cells are the result of the applied stress interfering with their biological processes.

Although our study managed to explore EcSSB LLPS dynamics in bacteria, we still try to understand the advantage of LLPS function in the case of EcSSB. Do they carry immediate advantages against stress, or do they only provide long-term benefits? Our group has already produced a variety of IDL-mutant EcSSBs that show modified or no LLPS properties. By generating cell lines exclusively expressing these modified proteins we try to answer this question in the near future. If it is proven that LLPS propensity provides a direct advantage for bacteria, it could pave the way for the development of new, broad-spectrum antimicrobial agents.

## Acknowledgements

I would like to thank my supervisor Dr. Mihály Kovács for the opportunity to get involved in this research during my master's degree and for his wisdom and expertise.

I would also like to thank my supervisor Dr. Péter Ecsédi, without his expertise, support and guidance this TDK research would not have been completed. His considerable involvement in the data evaluation and analysis was also fundamental during the completion of this essay.

I would also like to thank all members of Motor Enzymology Research Group at ELTE Department of Biochemistry for their valuable advice and for always being available for help and answering my questions. I would like to express my gratitude for Bálint Jezsó and Péter Ecsédi for developing the image evaluation scripts which were indispensable for data analysis.

I would also like to thank Dr. Edina Szabó-Meleg, Dr. Szilvia Huberné Barkó and Henriett Halász from University of Pécs, Department of Biophysics for the opportunity to use their SIM microscopes for visualization of our samples and their help in imaging.

Finally, I would like to thank my family and friends for their support during my studies.

## References

- [1] N. Chatterjee and G. C. Walker, “Mechanisms of DNA damage, repair, and mutagenesis,” Jun. 01, 2017, *John Wiley and Sons Inc.* doi: 10.1002/em.22087.
- [2] R. D. Shereda, A. G. Kozlov, T. M. Lohman, M. M. Cox, and J. L. Keck, “SSB as an organizer/mobilizer of genome maintenance complexes,” Sep. 2008. doi: 10.1080/10409230802341296.
- [3] G. M. Harami *et al.*, “Phase separation by ssDNA binding protein controlled via protein–protein and protein–DNA interactions,” 2020, doi: 10.1073/pnas.2000761117/-/DCSupplemental.
- [4] R. D. Shereda, D. A. Bernstein, and J. L. Keck, “A central role for SSB in Escherichia coli RecQ DNA helicase function,” *Journal of Biological Chemistry*, vol. 282, no. 26, pp. 19247–19258, 2007.
- [5] P. Handa, N. Acharya, and U. Varshney, “Chimeras between single-stranded DNA-binding proteins from Escherichia coli and Mycobacterium tuberculosis Reveal that their C-terminal domains interact with uracil DNA glycosylases,” *Journal of Biological Chemistry*, vol. 276, no. 20, pp. 16992–16997, 2001.
- [6] M. M. Cox, D. A. Soltis, Z. Livneh, and I. R. Lehman, “On the role of single-stranded DNA binding protein in recA protein-promoted DNA strand exchange.,” *Journal of Biological Chemistry*, vol. 258, no. 4, pp. 2577–2585, 1983.
- [7] T. C. Wang and K. C. Smith, “Effects of the ssb-1 and ssb-113 mutations on survival and DNA repair in UV-irradiated delta uvrB strains of Escherichia coli K-12,” *J Bacteriol*, vol. 151, no. 1, pp. 186–192, 1982.
- [8] B. Wang *et al.*, “Liquid–liquid phase separation in human health and diseases,” *Signal Transduct Target Ther*, vol. 6, no. 1, p. 290, 2021.
- [9] P.-H. Peng, K.-W. Hsu, and K.-J. Wu, “Liquid-liquid phase separation (LLPS) in cellular physiology and tumor biology,” *Am J Cancer Res*, vol. 11, no. 8, p. 3766, 2021.

- [10] C. A. Azaldegui, A. G. Vecchiarelli, and J. S. Biteen, “The emergence of phase separation as an organizing principle in bacteria,” Apr. 06, 2021, *Biophysical Society*. doi: 10.1016/j.bpj.2020.09.023.
- [11] X. Zhang *et al.*, “Emerging roles of liquid-liquid phase separation in liver innate immunity,” Dec. 01, 2024, *BioMed Central Ltd*. doi: 10.1186/s12964-024-01787-4.
- [12] N. J. Bonde, C. Henry, E. A. Wood, M. M. Cox, and J. L. Keck, “Interaction with the carboxy-terminal tip of SSB is critical for RecG function in *E. coli*,” *Nucleic Acids Res*, vol. 51, no. 8, pp. 3735–3753, May 2023, doi: 10.1093/nar/gkad162.
- [13] A.-M. Ladouceur *et al.*, “Clusters of bacterial RNA polymerase are biomolecular condensates that assemble through liquid–liquid phase separation,” *Proceedings of the National Academy of Sciences*, vol. 117, no. 31, pp. 18540–18549, 2020.
- [14] F. Heinkel *et al.*, “Phase separation and clustering of an ABC transporter in *Mycobacterium tuberculosis*,” *Proceedings of the National Academy of Sciences*, vol. 116, no. 33, pp. 16326–16331, 2019.
- [15] N. Al-Husini, D. T. Tomares, O. Bitar, W. S. Childers, and J. M. Schrader, “ $\alpha$ -Proteobacterial RNA degradosomes assemble liquid-liquid phase-separated RNP bodies,” *Mol Cell*, vol. 71, no. 6, pp. 1027–1039, 2018.
- [16] T. Zhao *et al.*, “Super-resolution imaging reveals changes in *Escherichia coli* SSB localization in response to DNA damage,” *Genes to Cells*, vol. 24, no. 12, pp. 814–826, Dec. 2019, doi: 10.1111/gtc.12729.
- [17] A. Schmidt *et al.*, “The quantitative and condition-dependent *Escherichia coli* proteome,” *Nat Biotechnol*, vol. 34, no. 1, pp. 104–110, 2016.
- [18] W. Yang, “Surviving the sun: repair and bypass of DNA UV lesions,” *Protein Science*, vol. 20, no. 11, pp. 1781–1789, 2011.
- [19] G. M. Harami *et al.*, “Shuttling along DNA and directed processing of D-loops by RecQ helicase support quality control of homologous recombination,” *Proceedings of the National Academy of Sciences*, vol. 114, no. 4, pp. E466–E475, 2017.

- [20] A. Singh and Y.-J. Xu, “The cell killing mechanisms of hydroxyurea,” *Genes (Basel)*, vol. 7, no. 11, p. 99, 2016.
- [21] N. S. Kosower and E. M. Kosower, “Diamide: An oxidant probe for thiols,” *Methods Enzymol*, vol. 251, pp. 123–133, 1995.
- [22] B. J. Juven and M. D. Pierson, “Antibacterial effects of hydrogen peroxide and methods for its detection and quantitation,” *J Food Prot*, vol. 59, no. 11, pp. 1233–1241, 1996.
- [23] E. Silva and T. Ideker, “Transcriptional responses to DNA damage,” *DNA Repair (Amst)*, vol. 79, pp. 40–49, 2019.
- [24] N. F. Shamsudin *et al.*, “Antibacterial effects of flavonoids and their structure-activity relationship study: A comparative interpretation,” *Molecules*, vol. 27, no. 4, p. 1149, 2022.
- [25] E.-S. Lin, R.-H. Luo, and C.-Y. Huang, “A complexed crystal structure of a single-stranded DNA-binding protein with quercetin and the structural basis of flavonol inhibition specificity,” *Int J Mol Sci*, vol. 23, no. 2, p. 588, 2022.
- [26] E.-S. Lin, Y.-H. Huang, R.-H. Luo, Z. Basharat, and C.-Y. Huang, “Crystal Structure of an SSB Protein from *Salmonella enterica* and Its Inhibition by Flavanonol Taxifolin,” *Int J Mol Sci*, vol. 23, no. 8, p. 4399, 2022.
- [27] S. Sengupta, M. S. Rahman, U. Mukherjee, J. Basak, A. K. Pal, and S. N. Chatterjee, “DNA damage and prophage induction and toxicity of nitrofurantoin in *Escherichia coli* and *Vibrio cholerae* cells,” *Mutation Research Letters*, vol. 244, no. 1, pp. 55–60, 1990.