Phase transition drives bacterial single-stranded DNA binding (SSB) protein mobilization during stress response and metabolic adaptation

Péter Ecsédi[1], Júlia Szittyai[1], János Pálinkás[1,2], Bálint Jezsó[1,2], Viktoria Katran[1], Zoltán J. Kovács[1,2], Henriett Halász[3], Tasvilla Sonallya[4], Tünde Juhász[4], Tamás Beke-Somfai[4], Szilvia Barkó[3], Edina Szabó-Meleg[3], and Mihály Kovács[1,2*]

[1] ELTE-MTA "Momentum" Motor Enzymology Research Group, Department of Biochemistry, Eötvös Loránd University, H-1117 Budapest, Hungary
[2] HUN-REN-ELTE Motor Pharmacology Research Group, Department of Biochemistry, Eötvös Loránd University, H-1117 Budapest, Hungary
[3] Department of Biophysics, Medical School, University of Pécs, H-7624 Pécs, Hungary
[4] Institute of Materials and Environmental Chemistry, HUN-REN Research Centre for Natural Sciences, Budapest H-1117, Hungary

* Correspondence: mihaly.kovacs@ttk.elte.hu

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Abstract

Single-stranded DNA-binding proteins (SSBs) are ubiquitous factors of genome metabolism, recently recognized for their ability to undergo liquid–liquid phase separation (LLPS). While *Escherichia coli* SSB (EcSSB) has emerged as a model for bacterial LLPS *in vitro*, its phase behavior and functional dynamics *in vivo* have remained unexplored. Here, we define the subcellular organization of EcSSB under diverse physiological and stress conditions using super-resolution microscopy coupled with newly developed image analysis tools. We demonstrate that EcSSB forms dynamic condensates during exponential growth, which disassemble in response to DNA damage, oxidative stress, antibiotic exposure, and metabolic adaptation. These phase transitions are attenuated in stationary-phase cells, indicative of reduced responsiveness. Moreover, we show that EcSSB is spatially segregated from nucleoid regions under stress-free conditions, but relocalizes to DNA-rich regions upon stress induction, supporting a regulated condensate-to-function transition. Our findings provide direct *in vivo* evidence for stress- and growth-phase-dependent modulation of EcSSB phase behavior, corroborated by biophysical characterization of EcSSB condensates bridging *in vitro* observations with physiological relevance. Our work establishes a quantitative framework for dissecting LLPS dynamics in bacterial cells and sets the stage for future strategies to target condensate-regulated pathways in antimicrobial development.

Main

Recent discoveries on liquid-liquid phase separation (LLPS) have transformed our understanding of cellular organization in eukaryotes. In contrast, the role of LLPS in bacteria remains poorly understood, despite growing evidence suggesting that phase transition processes play central roles in bacterial stress adaptation and post-stress resuscitation [1]. How bacterial cells detect and respond to stress—and whether LLPS constitutes a mechanistic basis for these processes—remains a fundamental scientific question. In this work, we combine super-resolution microscopy and quantitative image analysis to provide *in vivo* evidence that the essential genome maintenance factor, single-stranded DNA-binding (SSB) protein, forms condensates in *Escherichia coli* and that these phase-separated structures undergo stress- and growth-phase-dependent remodeling, correlating with DNA damage, metabolic state, and proliferation cues. SSB condensates were found to gradually coalesce and become more stable during metabolic slowdown, paralleling the earlier proposed "glass-like" transition of the bacterial cytoplasm during quiescent periods [2,3]. Our results support that SSB condensation contributes to cytoplasmic phase transitions, providing bacteria with a tunable way to modulate macromolecular mobility, sequestration, and release. Our findings uncover a direct link between protein phase behavior and bacterial stress adaptation, and open new avenues for targeting condensate dynamics in antimicrobial development.

Living cells must respond rapidly and effectively to DNA damage to mitigate its potentially severe impacts. Among others, ionizing and ultraviolet radiation, alkylating agents, and polycyclic aromatic hydrocarbons can cause exogenous DNA damage [4]. In addition, DNA is also vulnerable during physiological DNA metabolic processes including DNA replication, recombination, and repair. Nearly all genome maintenance pathways involve exposure of single-stranded DNA (ssDNA) segments whose processing is mediated by the action of single-stranded DNA binding (SSB) proteins in both prokaryotic and eukaryotic cells [5].

SSBs protect ssDNA from degradation and prevent its reannealing. In addition, SSBs recruit and organize genome maintenance complexes through protein–protein interactions (PPIs) [5]. *Escherichia coli* SSB (EcSSB), the prototypic SSB, is a homotetramer of 178-amino acid (19-kDa) subunits [6]. An EcSSB subunit comprises the N-terminal globular oligonucleotide/oligosaccharide-binding (OB) domain (amino acids (aa) 1-113) responsible for ssDNA binding [7], and an intrinsically disordered linker (IDL, aa 114-178) driving PPIs [8,9]. The C-terminal segment of the IDL (so-called C-terminal peptide, CTP, aa 170-178), a key PPI element, is highly conserved among bacteria [5,6,8,10,11]. It was recently demonstrated that EcSSB liquid-liquid phase separation (LLPS) is brought about by a fine-tuned interplay of the protein's different structural regions [12-14].

LLPS of proteins has been known for more than a century, yet its biological importance has only been recently recognized [15-18]. Numerous proteins have been reported to be capable of LLPS-based condensation [15,19], and LLPS has emerged as a key mechanism for the formation of membraneless organelles [1,20]. Examples for these structures in eukaryotes include Cajal bodies, P granules, and the nucleolus [18]. The formation of LLPS particles is typically driven by weak multivalent protein-protein and/or protein-nucleic acid interactions [21,22]. LLPS condensates serve a wide range of functions, such as buffering concentrations of proteins, rapid mobilization of biomolecules upon stress, and enhancing reactions by selectively concentrating molecules involved in biochemical processes [2,23]. Although the role of protein condensation in bacteria has remained less explored, it has recently become increasingly apparent that this phenomenon bears special importance within the spatial confines of bacterial cells lacking internal membrane-based compartmentalization [2,15]

The above recognition aligns with our previous *in vitro* finding that increasing concentrations of ssDNA dissolve EcSSB condensates in a stoichiometric fashion, suggesting a role for dynamic EcSSB decondensation in bacterial genome maintenance [12]. However, a comprehensive *in vivo* analysis of EcSSB condensate behavior has been lacking. A main challenge in studying bacterial condensates is the difficulty of visualization at this scale. Using super-resolution microscopy, Zhao *et al.* assessed the intracellular localization of EcSSB using an *E. coli* strain expressing a GFP-fused EcSSB variant from a genomic locus [24]. In unstressed state, EcSSB foci localized near the membrane and, to a lesser extent, at genomic sites. Treatment with the DNA damaging agent mitomycin C triggered the dissociation of EcSSB from the membrane, with nearly all EcSSB being found inside the cell. These indications, together with the ssDNA-gated *in vitro* condensation features of EcSSB [12-14,25], motivated us to perform a comprehensive, super-resolution microscopy-based definition of EcSSB subcellular patterns during bacterial growth, stress responses, and metabolic adaptations.

EcSSB forms dynamic condensates during growth, which consolidate upon growth arrest

To define the condensation behavior of EcSSB during bacterial growth, we recorded subcellular EcSSB patterns in exponential, stationary, and late ("death") growth phases using a well-characterized *E. coli* strain expressing EcSSB-GFP alongside the wild-type protein **(Fig. 1 and Table S1)** [24]. In the early exponential phase (OD_{600} = 0.2), cells exhibited weak EcSSB-GFP signals, with few faint granules observed. The low signal intensity was likely due to low protein expression and/or GFP maturation levels at this stage [26]. The majority of EcSSB granules (typically 1, but up to 6, per cell) appeared in the exponential phase (OD_{600} = 0.6). Notably, in late exponential and early stationary phases (OD_{600} = 0.6 + 1.5 h, + 3 h, and + 4.5 h), the number of granules in cells tended to consolidate into a single droplet, with cells lacking droplets becoming exceedingly rare. This trend shifted in later phases (OD_{600} = 0.6 + 7 h, + 10 h, and + 24 h) toward the dissolution of EcSSB granules. During these stages, the overall EcSSB-GFP signal diminished, indicating the onset of long-term stationary phase and cell death [27,28].

The time-dependent trend in EcSSB-GFP granule signal intensity indicates that the appearance of more intense granules is associated with reduced rates of division and correspondingly slower metabolism (*e.g.* OD_{600} = 0.6 compared to OD_{600} = 0.6 + 3 h, **Fig. 1**). Furthermore, as the number of granules decreased, their intensity increased, reflecting fusion of smaller condensates into a larger assembly (condensate maturation/coalescence; see also *in vitro* biophysical evidence described below). Based on EcSSB-GFP signal intensity analysis including all cells either harboring or lacking droplets, we detected a reduction in diffuse cytoplasmic EcSSB-GFP signal as cultures grew older, further indicating the assimilation of the dissolved EcSSB pool into condensates (eventually, up to 70 % of the total EcSSB-GFP signal became localized in condensate droplets) (**Fig. 1** and **Fig. S1**). We note that the high proportion of the cellular EcSSB-GFP signal in the granular form is also indicative of efficient incorporation of EcSSB-GFP into endogenous (wild-type) EcSSB droplets. During the death phase (OD_{600} = 0.6 + 10 h and 24 h), the overall cellular EcSSB-GFP signal declined, ultimately leading to the complete disappearance of droplets. Taken together, these observations provide evidence for our earlier proposition that EcSSB condensates act as protein reservoirs *in vivo*, sequestering EcSSB and likely its protein partners [12,25] and thus preventing unproductive DNA-modifying side reactions when the proteins are not required in large amounts at genomic sites.

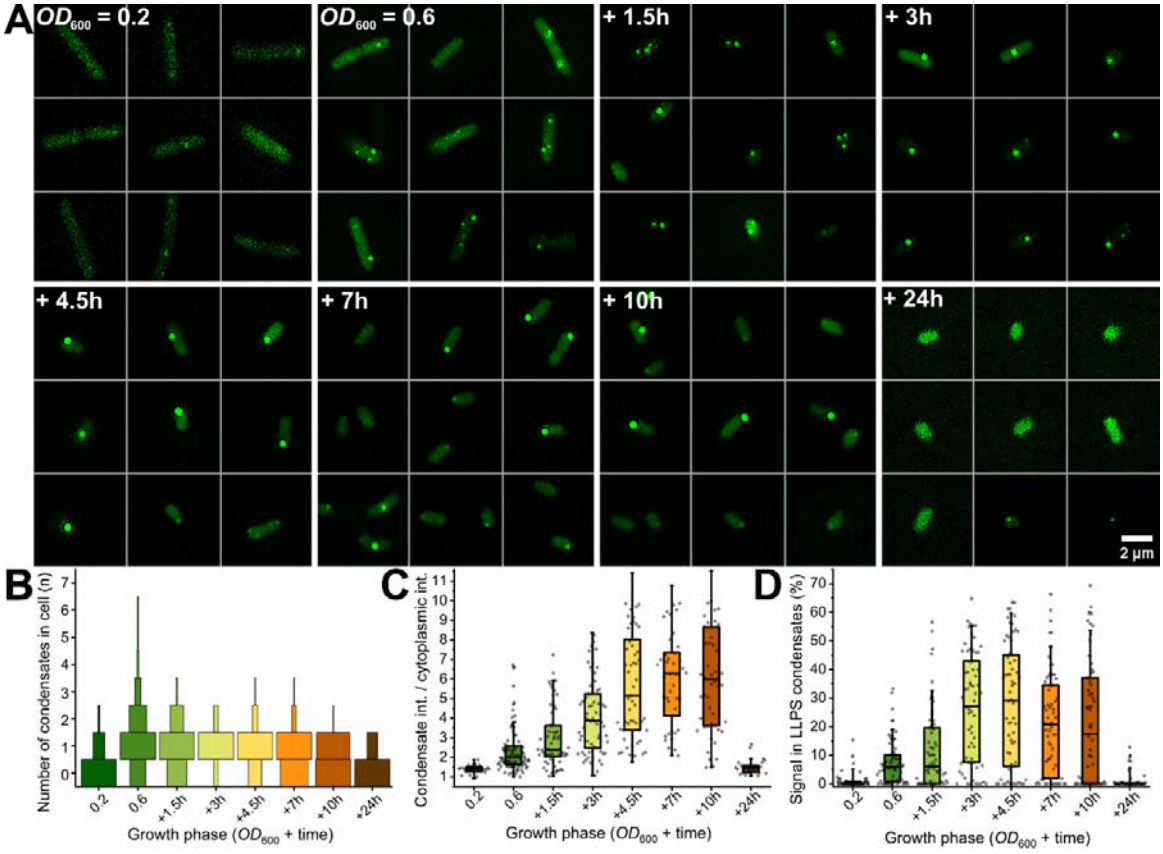

Figure 1. EcSSB shows gradual accumulation within cells during bacterial growth. (A) *Structured illumination microscopic (SIM) images of the EcSSB-GFP-expressing E. coli strain #29003* [24] *in different growth phases. Images were evaluated using a newly developed analysis methods described in* ***Supplementary file 1****. Data showing* ***(B)*** *the number of condensates per cell,* ***(C)*** *condensate intensities, and* ***(D)*** *the percentage of signal in condensates reveal successive accumulation of EcSSB from multiple granules into a single droplet during cell growth (n = 65 cells analyzed per condition, see also* ***Table S1****).*

EcSSB condensates disassemble under stress, but this process is attenuated in stationary-phase cells

Next, we analyzed the effects of a broad panel of stressors (described in **Table S2**, including dosages and mechanisms of action) on cells in the exponential growth phase (OD_{600} = 0.6). Of the applied agents, (*i*) hydroxyurea (HU) and NaCl suppress, while (*ii*) L-arginine (L-Arg), glucose, and lactose stimulate cell growth; (*iii*) UV irradiation, nitrofurantoin, and methyl methanesulfonate (MMS) directly damage DNA; (*iv*) diamide and H_2O_2 cause different forms of oxidative stress; (*v*) myricetin and taxifolin are antibacterial flavonoids interacting with prokaryotic SSBs (35054774, 35457218); and (*vi*) MES (2-(N-morpholino)ethanesulfonic acid, pH 6) was used to cause pH stress. Importantly, most applied stressors induced a decrease in the number and intensity of EcSSB condensates, with a concomitant increase in cytoplasmic EcSSB-GFP signal (**Fig. 2 and Table S3**). UV, L-Arg, glucose, H_2O_2, MES pH 6, myricetin, and taxifolin exerted the strongest effects. Upon these treatments, the most frequently observed number of condensates dropped to 0 (except in case of H_2O_2). Moreover, with a few exceptions, stressed cells contained less than 5 % of the EcSSB-GFP signal (if any) in condensate form, in contrast to the typical 10-20 % (maximum 40 %) in untreated cells (**Fig. 2D**). Notably, glucose, which is a more favored energy source for *E. coli* than lactose, exerted a

more pronounced EcSSB decondensation effect compared to the sugar alternative (**Fig. 2 and Table S3**). Taken together, these results provide evidence for stress-induced and metabolic change-induced EcSSB decondensation, supporting our earlier proposition based on EcSSB's *in vitro* condensation features (Harami 2020, Kovács 2025).

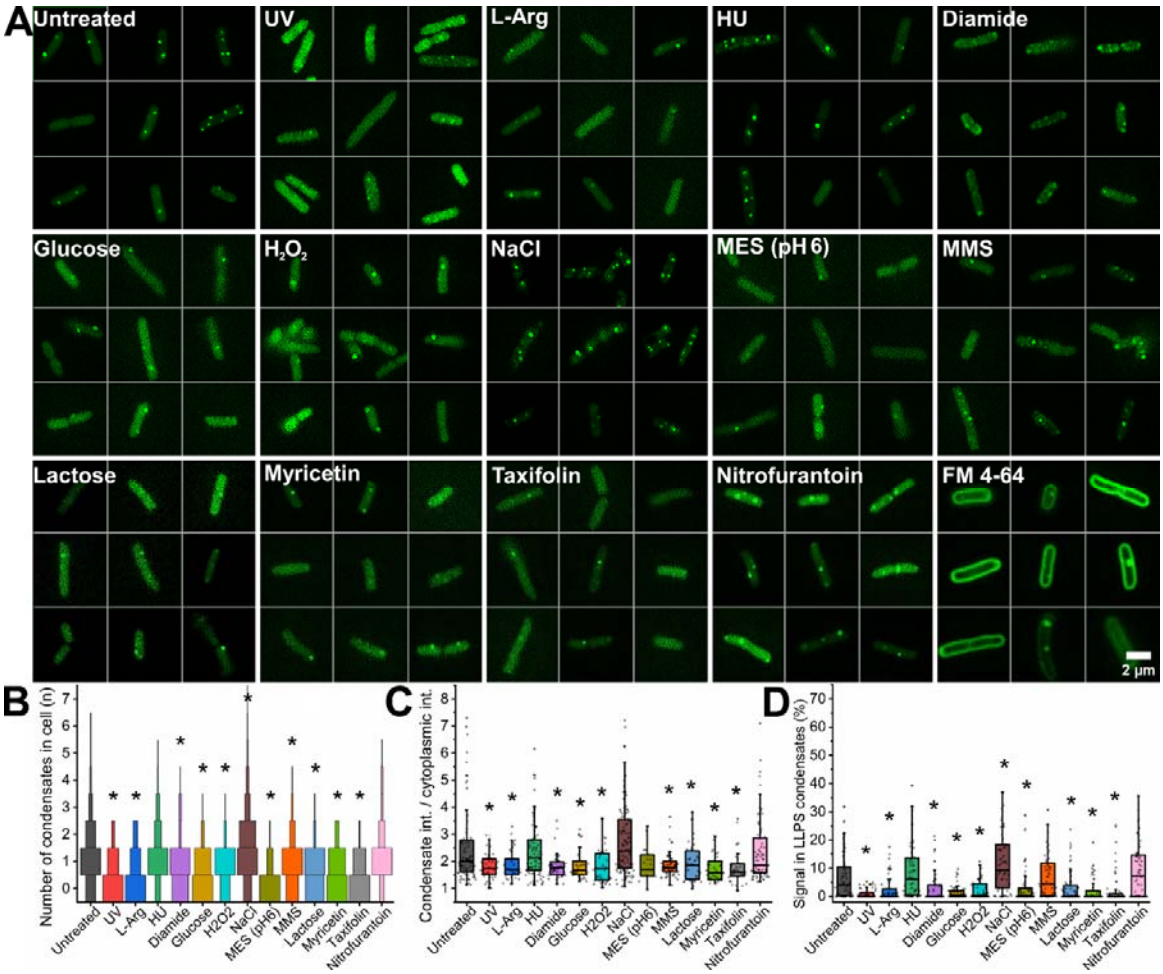

Figure 2. Various stressors induce EcSSB decondensation in exponential growth phase (OD_{600} = 0.6). (A) *Representative SIM images of E. coli strain #29003 show distinct EcSSB-GFP granules in untreated cells. Upon exposure to various stressors (as indicated; see* ***Table S2*** *for conditions), the EcSSB-GFP signal became dispersed throughout the cytoplasm, while* ***(B)*** *the number and* ***(C)*** *intensity of foci decreased, indicating dissolution of EcSSB condensates.* ***(D)*** *The reduction in the percentage of signal in granules further corroborates stress-induced condensate dissolution. Asterisks denote significant difference compared to untreated cells (Mann-Whitney test, $p < 0.05$, $n = 65$ cells analyzed per condition, see also* ***Table S3****). Cells treated with FM 4-64 were excluded from statistical analysis (see text).*

Other stressors including HU, MMS, nitrofurantoin, and NaCl, caused mild or no EcSSB decondensation effect (**Fig. 2 and Table S3**). Intriguingly, NaCl stress even enhanced EcSSB granulation, despite chloride ions being strong *in vitro* inhibitors of EcSSB condensation [12,14,25]. NaCl presumably causes osmotic changes in the cytoplasm, which could increase the local concentration of EcSSB, thereby driving condensate formation (see also biophysical results described below). HU was previously found to transiently inhibit DNA replication and repress DNA synthesis in *E. coli*, while the total amount of cellular DNA remained stagnant in the corresponding time period [29]. These features could explain the lack of effect of HU on EcSSB

condensation patterns (**Fig. 2 and Table S3**). The mild EcSSB decondensation effect of MMS could be due to the short exposure time applied [30,31]. Diamide had a special effect in causing EcSSB condensates to localize more toward the cell membrane, similar to that seen for untreated cells by Zhao *et al.* [24], in line with the observed interaction of EcSSB with membrane phospholipids in that study. Here we note that we also tested the effect of the FM 4-64 membrane dye, used by Zhao *et al.* [24], on EcSSB-GFP subcellular patterns. Intriguingly, we found that the dye caused EcSSB-GFP localization to membrane-adjacent cellular areas (**Figs. 2, 3, and S2**), and therefore we refrained from its use in other experiments. The possibility that the membrane-associated green signal could originate from FM 4-64 fluorescence bleed-through can be excluded based on (*i*) the complete disappearance of EcSSB-GFP signal from internal cellular areas; (*ii*) the lack of the EcSSB-GFP membrane localization effect at lower FM 4-64 concentrations (2 µM, as used by Zhao *et al.*) with detectable red fluorescence; (*iii*) the occasional presence of red fluorescent spots (FM 4-64 aggregates) showing no signal in the green channel.

Next, we determined the effects of the above-mentioned stressors during the stationary and death phases (OD_{600} = 0.6 + 3h (**Fig. 3 and Table S4**), OD_{600} = 0.6 + 24 h (**Fig. S2 and Table S5**), respectively). In the stationary phase, stressors elicited generally milder responses compared to the exponential phase. Nevertheless, UV, L-Arg, glucose, H_2O_2, and MES (pH 6) still elicited effective, albeit partial, EcSSB condensate dissolution in these phases. At the same time, an increase in the diffuse cytoplasmic EcSSB-GFP signal, along with the fragmentation of protein condensates, was evident, as reflected by the appearance of more numerous, less intense droplets. Glucose, lactose and, notably, HU were exceptions, as these agents primarily increased the cytoplasmic signal without significant condensate fragmentation. We attribute these findings to the applied sugars gradually reactivating DNA metabolic processes requiring free EcSSB, with a slow transformation, and UV, H_2O_2, and MES (pH 6) inducing more rapid and more pronounced disassembly of EcSSB condensates, leading to condensate fragmentation. HU stands out as it exhibited no significant effect during the exponential phase. In the stationary phase, however, HU could facilitate bacterial cell death, resulting in widespread dissolution of EcSSB condensates.

Unlike exponential-phase cells, stationary-phase cells retained up to 70 % of their EcSSB-GFP signal in the condensate form. However, in the presence of stressors including glucose, H_2O_2, HU, and UV, this value often dropped below 40 %. In addition, diamide exhibited a reduced propensity to induce localization of condensates near the membrane compared to that seen in the exponential phase, while UV occasionally promoted such localization (**Figs. 2 and 3**). The latter effect was not detectable in exponential-phase experiments, as UV completely dissociated EcSSB droplets in that phase (**Fig. 2**). Nonetheless, this UV-induced behavior was less pronounced in the stationary phase compared to diamide during the exponential phase. Together, these observations demonstrate that stationary-phase cells, exhibiting slower metabolism and lower demand for rapid DNA replication, show reduced sensitivity to stressors with respect to EcSSB condensation.

The subcellular localization of EcSSB underwent significant changes in the death phase (**Fig. S2 and Table S5**), with condensates becoming barely visible and only a faint cytoplasmic signal remaining. This phenomenon may occur because cells begin to reutilize their resources, while also degrading their EcSSB pool. Most stressors, except for NaCl, diamide, and nitrofurantoin, exerted minimal influence on EcSSB localization during this phase. These specific molecules promote the formation of small condensates, potentially by inducing

osmotic stress, which could lead to locally elevated protein concentrations, thereby promoting droplet formation.

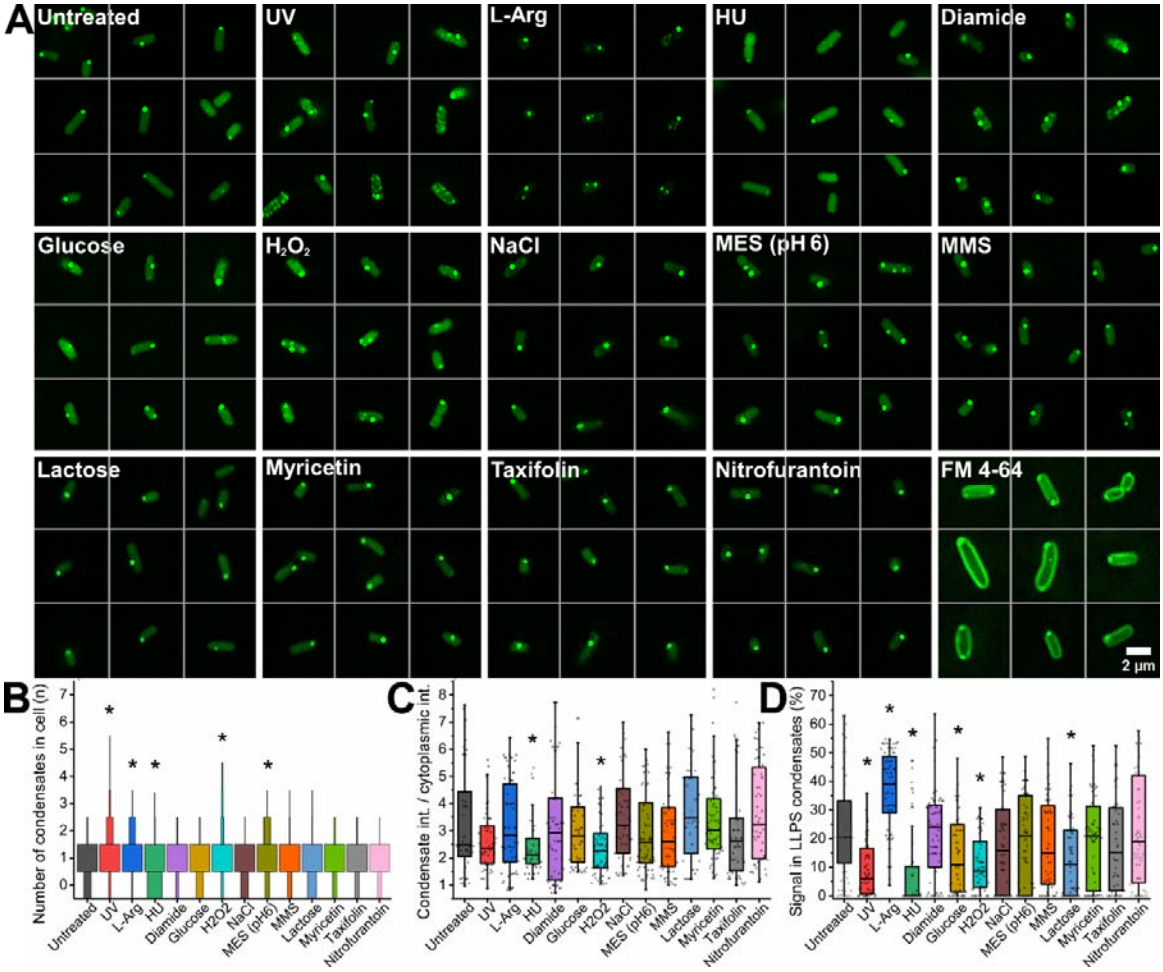

Figure 3. Stress-induced EcSSB decondensation is attenuated in stationary growth phase (OD_{600} = 0.6 + 3 h). (A) *Representative SIM images of E. coli strain #29003 show one bright focus in each cell, indicating EcSSB accumulation into a single large condensate. Stressors affected EcSSB-GFP patterns to a markedly lesser extent than in exponential-phase cells (OD_{600} = 0.6,* ***Fig. 2****; see* ***Table S2*** *for conditions). However, UV, MES (pH 6), H_2O_2, and glucose remained the most effective disruptors of EcSSB condensates. Analysis of* ***(B)*** *the number and* ***(C)*** *intensity of foci in cells showed that stressors causing DNA damage (e.g. UV and H_2O_2) elicit a rapid response initiating the fragmentation of EcSSB droplets, while glucose (presumed to restart cell growth over time) causes the droplets to slowly dissociate.* ***(D)*** *Cells contained markedly higher percentages of EcSSB in condensates compared to cells in earlier phases (OD_{600} = 0.2 and 0.6,* ***Figs. 1-2****). Asterisks denote significant difference compared to untreated cells (Mann-Whitney test, $p < 0.05$, n = 65 cells analyzed per condition, see also* ***Table S4****). Cells treated with FM 4-64 were excluded from statistical analysis (see text).*

EcSSB relocalizes to DNA-rich regions through stress-induced condensate dissolution

We previously demonstrated that ssDNA exerts an inhibitory effect on EcSSB condensation *in vitro* [12]. This observation raised the possibility that bacterial DNA (nucleoid regions) and EcSSB condensates do not colocalize in the cytoplasm in the absence of stress. To test this proposition and define the spatial relationship between DNA and EcSSB assemblies in cells, we

used Hoechst 33342 DNA dye and analyzed cells during exponential and stationary growth phases (**Fig. 4**). Indeed, we found that EcSSB-GFP granule and Hoechst signals were spatially separated in both growth phases, with the separation being more pronounced during the stationary phase. These findings provide evidence for separate localization of EcSSB condensates and nucleoid regions in the cytoplasm, especially when cells are in a resting state with less intense DNA metabolism. This result further corroborates the sequestering function of EcSSB condensates in stress-free physiological states.

Importantly, when H_2O_2 and MES (pH 6) were applied (selected based on the above results as effective stressors), the dissolution of EcSSB-GFP condensates was accompanied by a higher level of association between DNA and EcSSB-GFP signals (**Fig. 4**). These findings provide evidence for stress-induced DNA association of EcSSB *in vivo*, occurring through rapid dissolution of EcSSB condensates, as we proposed earlier based on EcSSB's *in vitro* condensation features [12].

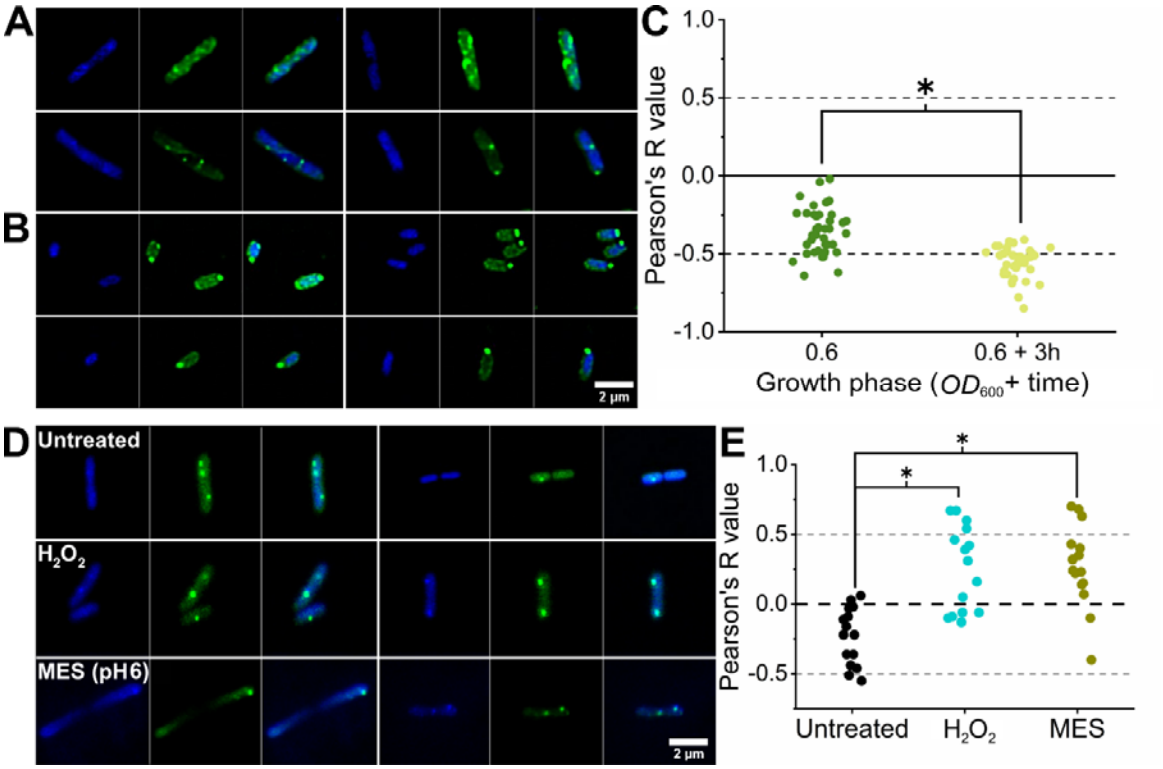

Figure 4. Physiological EcSSB sequestration from, and stress-induced association to cellular DNA. *Shown are SIM images of Hoechst 33342-stained (blue) E. coli #29003 cells (EcSSB-GFP, green) in the* ***(A)*** *exponential (OD_{600} = 0.6) and* ***(B)*** *stationary (OD_{600} = 0.6 + 3 h) growth phases.* ***(C)*** *Pearson's analysis of EcSSB-GFP and Hoechst signals shows marked anticorrelation in the exponential phase, which is significantly enhanced in the stationary phase in which genome maintenance processes are less intense (Mann-Whitney test, $p < 0.05$). These results support that EcSSB droplets function to sequester EcSSB from DNA during periods of lower genome metabolic demand.* ***(D)*** *Confocal microscopic images showing Hoechst 33342-stained (blue) E. coli #29003 cells (EcSSB-GFP, green) in the exponential growth phase (OD_{600} = 0.6) in the absence and presence of stressors (see* ***Table S2*** *for conditions).* ***(E)*** *Pearson's analysis of EcSSB-GFP and Hoechst signals shows the association of EcSSB with DNA under stress (Mann-Whitney test, $p < 0.05$). Note that DNA accumulates to*

a higher extent in larger protein droplets. This step presumably precedes the complete disassembly of condensates. Cells with H_2O_2 and MES treatment contained a smaller number of foci compared to stress-free cells, as was also seen in ***Fig. 2****.*

In vitro assays demonstrate physicochemical plausibility of the EcSSB liquid condensate-based cellular regulation mechanism

In addition to our *in vivo* experiments (**Figs. 1-4 and S1-2**), we carried out a comprehensive array of well-controlled *in vitro* biophysical measurements on EcSSB condensates to provide a quantitative framework that links droplet-level morphologies across isolated molecular systems and cellular organization (**Figs. S3-S7**). These *in vitro* fluorescence microscopic, turbidity-based, microscale thermophoresis (MST), microfluidic resistive pulse sensing (MRPS), and dynamic light scattering (DLS) experiments revealed that EcSSB condensation during exponential growth indeed could arise from protein concentrations surpassing a critical LLPS threshold, that cellular EcSSB granules represent *bona fide* LLPS assemblies, and that the applied stressors indeed modulate EcSSB condensates through cellular response mechanisms.

Discussion

In this work, we provide evidence for a dynamic condensation mechanism of *Escherichia coli* single-stranded DNA-binding protein (EcSSB), combining *in vivo* super-resolution microscopy with a comprehensive array of *in vitro* biophysical measurements. Our findings establish that EcSSB undergoes condensation in bacterial cells under stress-free conditions, and that the protein is rapidly mobilized to associate with genomic DNA via stress-induced decondensation. These observations provide support for an emerging context of prokaryotic DNA metabolism and stress responses involving dynamic protein condensation [2,32-34]. In **Figure 5** we integrated key current knowledge on the correspondence between *in vitro* effectors and the *in vivo* regulation of EcSSB condensation dynamics. Global cytoplasmic transitions from fluid-like to glass-like states have been reported in response to decreased metabolic activity [2], and metabolism-driven cytoplasmic fluidization has been linked to enhanced molecular mobility [2,3]. Our findings on growth phase-, stress-, and metabolism-dependent EcSSB condensation suggest that these mechanisms could contribute to this broader cytoplasmic phase transition during stress and metabolic adaptation.

Importantly, we demonstrate that EcSSB condensates dissolve or fragment in response to various stressors, and that EcSSB condensates are spatially separated from DNA in stress-free cells, but show increased DNA association upon stress. These results support our previous hypothesis that LLPS-mediated sequestration of EcSSB serves as a reservoir that can be rapidly mobilized upon stress-induced demand [12]. Supporting this model, we previously showed that EcSSB's interacting partner, RecQ helicase, becomes enriched within EcSSB condensates, suggesting co-sequestration of genome maintenance factors [12,25]. This mechanism could serve to prevent unproductive protein-DNA interactions at high protein concentrations, thereby preserving genomic integrity. Additionally, sequestration could protect sensitive factors from degradation, reducing energy consumption and enhancing survival. Our findings also raise the possibility that EcSSB's LLPS propensity confers a selective advantage to bacteria *via* enhanced stress survival. The tools established in the current work enable future testing of this proposition *via* the use of genetically engineered EcSSB variants that are unable to undergo LLPS but retain their ssDNA-binding function [25].

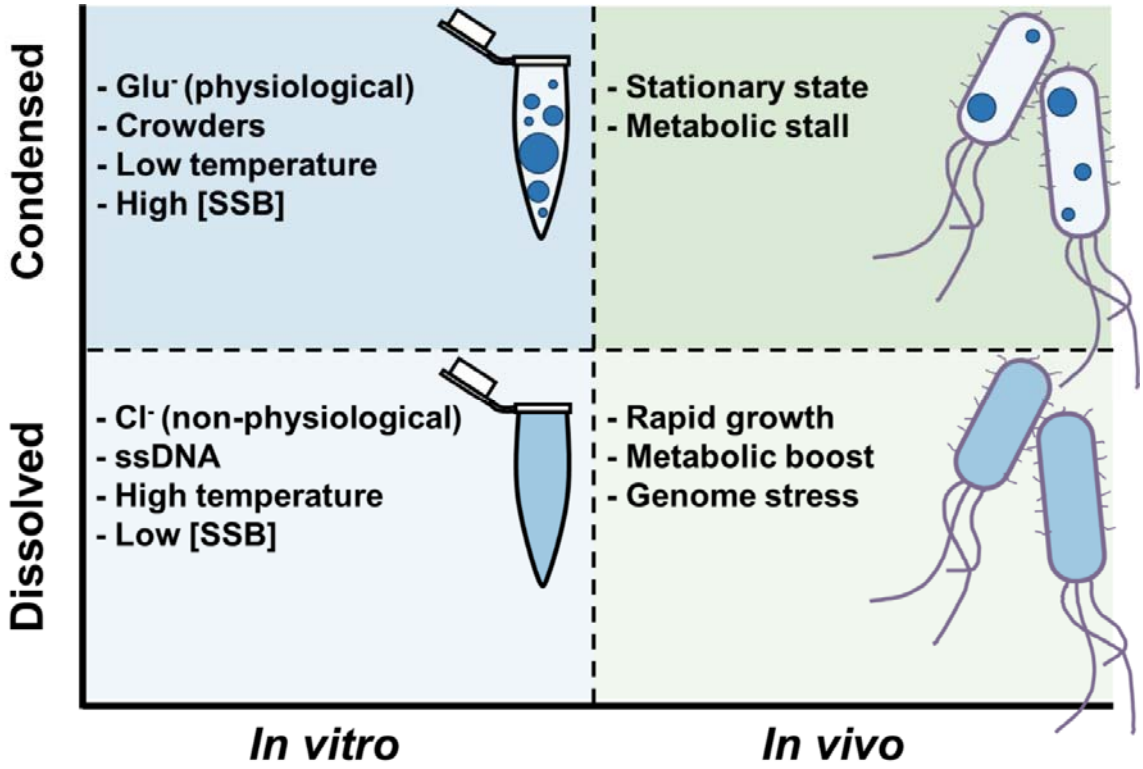

Figure 5. "Phase map" of EcSSB. *EcSSB condensates are highly dynamic structures whose formation and dissolution is regulated by multiple factors both in vitro [12,14,25] and in vivo (this paper). This responsiveness allows EcSSB condensates to function as efficient tools for bacterial cells, facilitating the sequestration of proteins when not required and enabling their rapid mobilization to sites of action upon demand. Note that in vitro conditions mimicking the unstressed cellular environment promote condensation.*

During super-resolution imaging, we observed EcSSB foci with diameters typically ranging from 100–500 nm. This size distribution is consistent with estimated cellular concentrations of EcSSB [35]. Based on the crystal structure of EcSSB (PDB: 4MZ9) in which EcSSB forms a tetrameric assembly with a ~50 Å diameter, a few hundred tetramers packed in a crystalline-like arrangement would result in condensate sizes of approximately 50–200 nm. However, we note that this estimation does not account for differences in packing density between crystals and LLPS droplets, the structural effects of EcSSB's intrinsically disordered tail region, and the possible incorporation of EcSSB's interacting partners, all of which could influence condensate size *in vivo*.

Finally, we emphasize that the imaging analysis pipeline defined in the current study (**Supplementary file 1**) constitutes a valuable tool for the broader study of LLPS *in vivo* by enabling quantitative assessment of condensate properties in diverse genetic or environmental contexts. Taken together, our work positions EcSSB condensation as a dynamic and tunable component of bacterial DNA metabolic regulation. Hence, these insights can also open potential avenues for antimicrobial discovery by targeting phase separation pathways.

Materials and Methods

Materials

Stressors used in this study, including their applied dose and mechanism of action, are described in detail in **Table S2**. In this work we used an *E. coli* strain #29003 expressing a GFP-fused EcSSB (EcSSB-GFP) from a genomic locus, which was a kind gift from Piero R. Bianco [24]. Recombinant EcSSB expression and purification, as well as AF555 (AlexaFluor 555) and IAF (5-iodoacetamidofluorescein) labeling was carried out as described in Harami *et al*. [12] Since EcSSB does not contain any cysteines, the $EcSSB^{G26C}$ variant was used for IAF labeling. AF555 labeling was performed on wild type EcSSB. EcSSB protein concentrations are expressed in terms of EcSSB tetramers throughout this article.

Cell culture preparation for fluorescence imaging

In this work, we used *E. coli* strain #29003, expressing GFP-fused EcSSB (EcSSB-GFP) from a genomic locus, as a kind gift from the Bianco group [24]. Importantly, the sequence coding for EcSSB-GFP was introduced downstream (not in place) of the wild-type (WT) EcSSB coding sequence in this strain. Consequently, these bacteria express both WT EcSSB and EcSSB-GFP. Thus, the fused construct can be utilized for fluorescence-based detection, while the WT protein provides intact SSB functionality in the cells.

Cells were grown overnight in Luria–Bertani medium (LB) containing kanamycin (25 µg/ml) and chloramphenicol (34 µg/ml) at 37°C. 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_{600} value + time indicating the different growth phases (exponential, stationary or death phase). We 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 $MgSO_4$ twice, then resuspended in $MgSO_4$ containing the stressors at concentrations indicated in **Table S2**. The pH of stressor solutions was adjusted to 7.0 except for MES (pH 6) when necessary. Cells were shaken for 30 minutes at 200 rpm at 37°C and subsequently imaged. UV-stressed cells were observed without incubation to avoid regeneration. Depending on cell density, dilutions were applied before imaging to avoid a confluence of cells during imaging.

Super-resolution (SIM) and confocal microscopy of *E. coli* cells

For SIM microscopy, Zeiss High Performance 0.17 ± 0.005 mm thick glass coverslips and a Zeiss Elyra S1 SIM microscope with 488-nm (Lasos) laser were used. Hoechst 33342 (Biotium, #40046) DNA stain images were captured using a Nikon X-Light V3 Deep SIM microscope (LED, solid state from Lumencor). Cells were treated with Hoechst for one minute followed by two wash steps before observation.

For confocal microscopy, a Zeiss LSM710 microscope was used with Menzel-Glaser (20x20 mm, Thermo scientific) glass cover slides. Hoechst 33342 staining was performed as described for SIM imaging.

Evaluation of microscopic images of *E. coli* cells

Image analysis was performed using Fiji software. For image evaluation purposes, two scripts were developed for automatic quantification of protein condensates in cells **(Supplementary file 1)**. During evaluation, 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 foci (condensates) using a modified Find Maxima function (**Fig. S8, Supplementary file 1**). To determine the percentage of fluorescent signal present in the condensates compared to the cytoplasm, the developed script (**Supplementary file 1**) was used to outline condensates and to subtract them from the original image. The difference between intensities was used to define material percentage within condensates (**Fig. S1**). Colocalization analysis of images of Hoechst-stained cells was performed using Fiji plugin Coloc2.

General measurement conditions for *in vitro* experiments

In vitro measurements were performed at 25°C in LLPS buffer containing 20 mM Tris-acetate (Tris-OAc) pH 7.5, 5 mM MgOAc and 50 mM NaGlu.

Turbidity assays of purified EcSSB condensates

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 µL, in a buffer containing 20 mM Tris-OAc pH 7.5, 5 mM MgOAc and 50 mM NaGlu. The EcSSB tetramer concentration was 5 µM. Measurements were performed in triplicate at room temperature.

Epifluorescence microscopy of purified EcSSB condensates

The effects of stressors on EcSSB condensates *in vitro* were visualized using epifluorescence microscopy. Samples contained 5 µM EcSSB tetramers and 0.1 µM fluorescein-labeled EcSSB, which readily became enriched in EcSSB droplets [12]. Samples were measured on Ibidi 15 Well µ-Slide Angiogenesis slides at room temperature. A Nikon Eclipse Ti-E TIRF microscope was used in epifluorescence mode with a Cyan 488-nm laser (Coherent). Determination of total droplet area in middle ROI was carried out as in [36].

Microscale thermophoresis (MST)

The thermophoretic mobility of 5 µM EcSSB (containing 50 nM iodoacetamidofluorescein (IAF)-labeled $EcSSB^{G26C}$) was monitored using a Nanotemper Monolith device (set to 20 % MST power and 75 % excitation power) in LLPS buffer. Premium capillaries were used for sample loading. Capillary scans showed ideal capillary shapes and no protein adhesion to the surface. Baseline was recorded for 5 seconds before infrared irradiation for 30 seconds. Fluorescence recovery was monitored for additional 5 seconds after the end of irradiation. Fluorescence was normalized to baseline. Thermophoretic mobility was determined as the fraction of the baseline fluorescence signal (F_{norm}) measured at 20 seconds irradiation.

Microfluidic resistive pulse sensing (MRPS)

MRPS measurements were performed using an nCS1 instrument (Spectradyne LLC, USA). Data obtained were analysed using Viewer software (Spectradyne LLC, USA). EcSSB was diluted in LLPS buffer unless otherwise indicated. Samples were measured using a C-2000 cartridge (~250 nm to 2000 nm measurement range).

Dynamic light scattering (DLS)

EcSSB samples were diluted in LLPS buffer and measured in low volume disposable cuvettes with 1 cm path-length (UVette, Eppendorf Austria GmbH) using a W130i dynamic light

scattering device (DLS, Avid Nano Ltd., High Wycombe, UK) equipped with a diode laser (660 nm) and a photodiode detector.

Data Availability

The data underlying this article are available in the article and in its online Supplementary Material. The data underlying this article will be shared upon reasonable request to the corresponding author.

Supplementary Data

Supplementary Data are provided with this article.

Author Contributions

P.E.: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology Validation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **J.S.**: Investigation, Data curation, Validation, Writing – original draft. **J.P.**: Investigation, Methodology. **B.J.**: Conceptualization, Methodology. **V.K.**: Investigation. **Z.J.K.**: Investigation. **H.H.**: Investigation, Methodology. **T.S.**: Investigation. **T.J.**: Investigation, Methodology. **T.B.S.**: Conceptualization, Methodology. **S.B.**: Investigation, Methodology. **E.S.M.**: Investigation, Methodology. **M.K.**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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