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A Review

Newly-discovered behaviour in the bacterial histone-like protein, HU

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This paper summarizes the contents of a talk delivered at the MS University of Baroda (Vadodara, Gujarat) on 3rd March, 2023, at a conference held to celebrate proteins in commemoration of the birth centenary of Prof. G. N. Ramachandran. Here, we review several recent discoveries and applications from our group that relate to HU, a DNA-binding nucleoid-associated protein found in bacteria: (1) HU uses its DNA-binding sites to bind to lipopolysaccharide (LPS) upon bacterial cell surfaces, in the extracellular milieu in biofilms, thus working as a glue to attach bacteria to extracellular DNA; (2) HU and DNA perform mutual macromolecular crowding, as well as mutual charge neutralization, to together undergo condensation into nucleoids that appear to maintain DNA in a compacted state in bacterial genomes through liquid-liquid phase separation (LLPS); (3) HU appears to have evolved to avoid use of the amino acid residue, tryptophan, to avoid damage to bacterial genomic DNA by a combination of sunlight and photosensitized oxidation by tryptophan photodecomposition products; (4) HU's N-terminal (positive) charge destabilizes hydrophobic inter-subunit interactions between beta strands in HU dimers and promotes subunit exchange between HU-A and HU-B (the two isoforms of HU in gut bacteria), thus hindering the facile dissociation of subunits if an N-terminal 6xHis affinity tag is present; (5) HU-A and HU-B can be genetically fused to generate a simulacrum of an HU heterodimer; (6) HU's DNA-binding regions from two bacterial homologs (one mesophilic and the other thermophilic) can be isolated and genetically fused to generate a novel thermostable DNA-binding protein; (7) HU's ability to titrate onto the bacterium's nucleoid can be exploited to deploy fluorescent protein-labelled HU in cells to test for leaky expression from bacterial promoters, using fluorescence microscopy.

Keywords: Biofilms, DNA compaction, DNA condensation, DNA-binding protein, Histone-like protein, HU, Leaky gene expression, Liquid-liquid phase separation, Nucleoid associated proteins (NAPs), Protein engineering, Protein evolution, Tryptophan photooxidation

Introduction

DNA generally exists within chromosomes in a state in which it remains bound to one or more DNA-binding proteins that lack sequence-specificity andare abundant. In bacteria, chromosomes exist in direct contact with the bacterial cytoplasm, since there is no nuclear envelope. The chromosome in a bacterium thus serves as a proxy for the cellular nucleus. It is often referred to as the bacterium's "nucleoid"[1]. Seven different types of DNA-binding proteins, collectively known as Nucleoid Associated Proteins (NAPs), are involved in the packaging of DNA within the chromosome of the model bacterium known as *Escherichia coli*[2]. One of these seven types of proteins is a histone-like protein known as HU. In *E. coli*, HU exists in two isoforms that are called HU-A and HU-B. Homologs of these two isoforms are also seen in several other bacteria that exist in the gut; however, most bacteria possess a single isoform of HU[3]. HU-A and HU-B are basic proteins (with pI values falling between 9.0 and 10.0). They are small in size (~ 90 amino acids long), and exist either as homodimers (HU-A/HU-A or HU-B/HU-B) or as heterodimers (HU-A/HU-B). While little is known about the extent to which HU dimers associate further *in vivo* in the context of the nucleoid, sub-populations of HU-A are known to form tetramers *in vitro*, while sub-populations of HU-B are known to form tetramers as well as octamers, suggesting that HU-B shows a greater tendency to oligomerize *in vitro* than HU-A[4]. The existence of these two isoforms, HU-A, and HU-B, has been known for decades. Much is also already known about how their relative proportions vary as a function of growth phase in culture, and about how homologs of HU from different bacteria bind to defined pieces of DNA,to cause DNA to become bent through such binding, through the use of techniques such as X-ray crystallography, gel electrophoresis and various forms of spectroscopy[5].

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We have been interested in HU for several years now. Although our interest in HU began with an examination of HU to explore the scope for transforming it from a non-sequence-specific DNA binding protein into a library of sequence-specific DNA-binding variants, we never did get around to building such a library. Instead, we became interested in understanding the different modes of DNA binding by wild-type HU-A and HU-B. Thus, we cloned HU and introduced tryptophan residues in place of phenylalanine residues in the neighbourhood of its DNA-binding regions, to try and develop variants that alter their spectroscopic (fluorescence) properties upon binding to DNA (note: wild-type HU-A and HU-B possess neither tryptophannor tyrosine residues). So, as things turned out, we ended up doing (and discovering) much else in respect of *E. coli* HU by following up various stray observations and the hypotheses that arose from such observations. This article, derived from a talk delivered at a conference celebrating proteins in commemoration of the birth centenary of Prof. G. N. Ramachandran is about some of the recent work that we have carried out with HU. The work has produced numerous unanticipated insights into the life-cycle and behaviour of this otherwise rather-staid non-sequence-specific DNA binding protein. In each of the following sections below, we briefly summarize one new insight, or application, that involves either HU-A or HU-B or both. In each section, a reference is provided to a publication from our group in which every other relevant detail regarding the work may be found. The sections are also summarized in (Fig. 1).

In biofilms, HU binds to outer membrane lipopolysaccharide (LPS) and stabilizes the embedding of bacteria within an extracellular DNA-based matrix[6]

When bacteria die through lysis, they disgorge their contents into the surrounding medium. A large fraction of what gets disgorged into the extracellular medium is the bacterium's DNA. This DNA is then called extracellular DNA (or eDNA). This eDNA constitutes the major component of the extracellular polymeric substance (EPS) in bacterial biofilms. Indeed, eDNA is such an integral component of biofilms that treatment with DNAse dissociates biofilms to release component bacteria[7]. The presence of bacteria in a DNA-dominated environment raises a physico-chemical conundrum that requires to be addressed for completeness of understanding of how bacteria come to be embedded in DNA. The surfaces of bacteria are always negatively-charged, on account of the presence of either lipopolysaccharide (LPS) or lipotechoic acid (LTA) molecules upon their surfaces. These contain sugar-phosphate groups. DNA is also negatively charged, on account of the sugar-phosphate groups that are present in the DNA backbone. Thus,

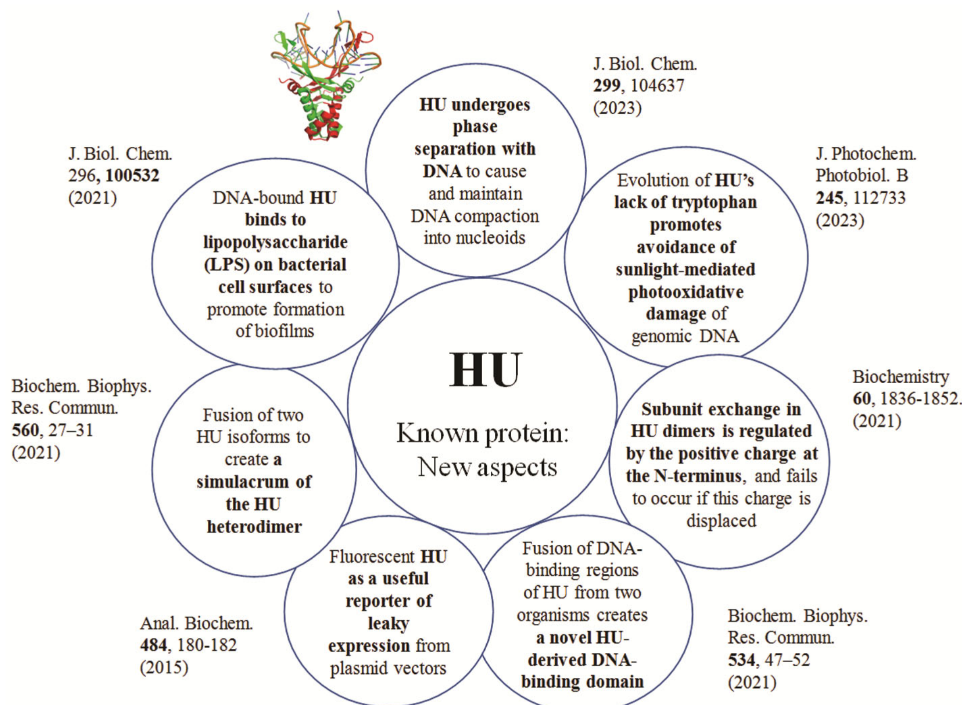

Fig. 1 — Seven discoveries and/or applications concerning HU from our research group

repulsions must occur between the DNA and the bacterial surfaces, both of which are negatively-charged, unless there is a 'bridging' molecule bearing positive charges which binds to both, to neutralize the charge-charge repulsions between bacteria and DNA. We recently showed that HU (which is a DNABII class protein) is the bridging molecule which functions as a glue joining bacteria to DNA in biofilms[6].

We found that HU binds to sugar-phosphate groups present in LPS through the same sites upon HU's surface that are involved in HU's binding of DNA. We then showed that this allows HU to become capable of either (a) clumping negatively-charged bacteria through HU-LPS contacts made by HU multimers, or (b) facilitating biofilm formation by allowing bacteria to bind to HU-bound DNA, through the use of unoccupied DNA-binding sites upon HU. Salt and pretreatments of various kinds were shown to neutralize the scope for clumping of cells and the binding of cells to HU. Our work thus established that HU is the glue that actually holds bacterial biofilms together. This explains why HU is found in biofilms along with DNA, as one of the most abundant of all proteins found in biofilms[8]. Our work also explains why antibodies against DNABII proteins (specially, anti-HU antibodies) are reported to be capable of causing the disintegration of biofilms. The studies conducted for this work were supported by different kinds of spectroscopy, spectrometry, cytometry, and microscopy, as well as methods such as microscale thermophoresis, biolayer interferometry and changes in birefringence in liquid crystals coated with LPS, upon binding of HU.

In the cell, HU compacts and condenses DNA through liquid-liquid phase separation (LLPS) aided by charge-neutralization, mutual-crowding and de-rigidification of the DNA double helix[4]

As already mentioned, bacterial cells contain DNA in the form of a nucleoid. In this nucleoid, in the bacterium known as *E. coli*, DNA exists in a state of extreme (2,000- to 4,000-fold) compaction. The *E. coli* genome reduces from an extended length of 2000 μM (as B-DNA) to a length of less than 0.5 μM in any direction, to fit inside a rod-shaped *E. coli* cell with a length of ~1 μM and a diameter of ~0.5 μM. Remarkably, all transactions involving DNA, including replication and transcription, are required to occur in this state of compaction, without scope for all of the nucleoid's DNA to become un-compacted at any one time, because there is no space for DNA to do so inside a growing and dividing *E. coli* cell. This indicates the need for the existence of a generic mechanism for DNA compaction. Of course, DNA-binding and the supercoiling of DNA by topoisomerases does achieve a certain level of compaction, through the looping, bending, bunching, wrapping and bridging of different sections of DNA. However, none of these inspires the confidence that they could either ensure compaction at all times, or comprise a generic mechanism for the perpetuation of the compacted state through generations *ad infinitum* in a growing population of bacteria.

There have been speculations that nucleoids can potentially exist in a state of phase separation from the cytoplasm; however, neither have the details of such phase separation ever been worked out, nor has phase separation been previously proposed to be the primary mechanism by which DNA is always kept compacted and in a state of separation from the cytoplasm through the non-sequence-specific accretion of DNA by any protein(s). Against this background, we recently showed that addition of HU induces accretion and compaction of DNA into liquid-liquid phase-separated (LLPS) condensates[4]. When HU is mixed with DNA under entirely physiological conditions of temperature, ionic strength and pH, and using physiological concentrations of DNA base pairs and HU, both DNA and HU neutralize each other's charges and this presumably triggers a de-rigidification of the DNA double helix, thus reducing its persistence length. We found that addition of HU to DNA led to the immediate and spontaneous formation of condensates consisting of HU-complexed DNA. We demonstrated that this occurs without the use of any devices/agents (*e.g.*, through an excluded volume effect) to increase the local concentrations of either DNA or HU protein, simply through mutual macromolecular crowding of DNA and HU protein by each other. We demonstrated that this occurs with many forms of nucleic acids, including single-stranded DNA, double-stranded DNA, cruciform DNA, sheared genomic DNA, and RNA. Interestingly, we also found that HU-B is better at promoting liquid-liquid phase separation of DNA than HU-A, and demonstrated that this owes to differences in their tendency to form multimers, using various protein engineering experiments. Our findings thus suggest an explanation for the use of HU-A by

actively growing and dividing cells and the use of HU-B by more mature cells that divide less frequently.

Furthermore, we also showed that a different abundant and non-sequence-specific DNA-binding NAP called Dps, which is naturally overexpressed in the stationary phase of *E. coli*, is also capable of condensing DNA, like HU. We showed that DNA, HU and Dps form heterotypic multiphasic condensates that are suggestive of mechanisms which enable the gradual transformation of nucleoids from a liquid-liquid phase separated state towards greater solid-like behaviour, as cells enter stationary phase. Interestingly, in multiphasic condensates of DNA, HU and Dps, we found that DNA is preferentially titrated into Dps-enriched regions.

We also used RFP-tagged HU to demonstrate LLPS behaviour in nucleoids *in vivo* using super-resolution fluorescence microscopy. Our work thus shows that LLPS of DNA caused by NAPs is the necessary for maintenance of bacterial genomic DNA in the compacted state called the nucleoid[4].

During evolution, HU appears to have avoided using tryptophan in its sequence to avoid tryptophan photoproduct-mediated oxidative damage to the genome[9]

Tryptophan (Trp) is a unique aromatic amino acid residue, with an indole moiety for a side chain. This moiety absorbs UV-B light and decomposes through indole ring breakage into a series of photosensitizers such as N-formylkynurenine (NFK), kynurenine (KYN), and 3-hydroxykynurenine (3OHKYN), which display progressively longer wavelengths ofmaximal absorption in the UV-A and in the visible regions of the electromagnetic spectrum[10]. Such decomposition of Trpis a low probability event, with the higher probability remaining that of normal absorptive (excitative) and emissive behaviour of the indole moiety. Therefore, such decomposition is not very important for proteins that are rapidly turned over, *e.g.*, on a timescale of a few minutes or a few tens of minutes. This is because Trp-derived photo-sensitizers in such proteins never really get the chance to either form, or to give rise to other photosensitizers (down the photo damage cascade) since the protein itself gets quickly degraded and replaced before such photosensitizers happen to be created. Notably, damage and decomposition of Trp can occur (i) through either light-mediated photooxidation (in cells that are exposed to light), or (ii) through the direct oxidation of side chains by reactive oxygen species (ROS) within biological systems.

While the photooxidative decomposition of Trpis unimportant for most proteins, it can assume great importance in proteins that have very long lifetimes, *e.g.*, on a timescale of tens or hundreds of hours, in short-lived organisms, or tens or hundreds of days, in long-lived organisms. In our opinion, photooxidative damage to Trp and/or oxidative damage to Trp (mediated by ROS in the cytoplasm) can constitute a major problem for proteins of two types: (i) proteins that exist within cells that lack all protein turnover machinery [*e.g.*, the crystallins of the vertebrate eye lens, which lack scope for metabolic turnover because their host cells have dispensed with cell nuclei, DNA, RNA, replication, transcription and translation during development and differentiation, as in the case of vertebrate lens epithelial cells in which crystallins exist for decades after they are produced]; (ii) proteins that exist in sub-cellular locations that remain largely inaccessible to protein turnover machinery for long lengths of time [*e.g.*, histones and NAPs that have become buried away from the cytoplasm, and from cytoplasmic arrangements for protein degradation and replacement (through fresh synthesis), through burial within DNA; especially in regard of proteins with a basic isoelectric point (pI), since acidic DNA can completely envelop such proteins and sterically prevent access to any components of protein turnover machinery].

In the case of the lens crystallins, which fall in the first of the above categories, much is known about how the crystallins accumulate damage over decades (and also cause further damage to other proteins and lipids through photosensitized oxidation), with all of this eventually leading to protein precipitation within lens cells, and to the scattering of incident light by lenses when they form cataracts. However, in the case of histones and NAPs, we found that the subject of any photo damage potentially occurring to Trp to create further damage to either DNA or other chromosomal proteins has remained completely unexplored, to date. In fact, even the relevant questions in this regard have not yet been framed. So, we performed a bioinformatics-based analyses of all known relevant sequences of DNA-binding proteins: across multiple species of bacterial NAPs; across histones in eukaryotes; as well as across other DNA binding proteins and transcription factors in humans. We discovered that all proteins possessing a basic isoelectric point (pI) that are involved in the

packaging of DNA lack Trp in their sequences, as we would have predicted. In contrast, we found that DNA packaging proteins with acidic pI values do contain Trp residues, as indeed do all other DNA-binding proteins that interact transiently with DNA, such as transcription factors, and not semi-permanently (like HU or histones). We rationalized this by arguing that NAPs with a basic pI and histones (which also all have basic pI values) have the propensity to become completely buried by DNA and, therefore, inaccessible to protein turnover machinery. We argued that, in all likelihood, this lack of access makes it risky for such proteins to possess any Trp residues (especially in cells that happen to be exposed to sunlight, such as skin cells or the cells of unicellular organisms). This is because light could potentially cause photo damage in Trp residues, with the lack of rapid protein turnover allowing accumulation of NFK, KYN and 3OHKYN in Trp-containing NAPs and histones, leading to deleterious effects for these proteins and all associated DNA.

We proposed that the production of ROS such as singlet oxygen or hydroxyl radicals could cause damage to DNA which would not be immediately repaired either, because just as proteins buried within chromosomes cannot be accessed by turnover machinery, DNA in the interior of a chromosome too is unlikely to be accessible to DNA repair machinery. Thus, it would appear that evolution has organized for such proteins to contain only the other nineteen naturally-occurring residues, and not tryptophan[9]. So, we created a DNA-binding mutant of wild-type Trp-lacking HU-B, by replacing the phenylalanine residue at position 47 in its sequence by the residue, Trp. This mutant (HU-B F47W) was then found by us to accumulate both photosensitizers such as NFK, KYN and 3OHKYN, as well as photooxidatively-derived covalent crosslinks inside the HU-B protein, upon initial exposure to UV-B, and subsequent exposure to UV-A and visible light, *in vitro*. In contrast, this behaviour was not seen in wild-type HU-B that lacks Trp residues. Then we found that bacterial cells expressing the HU-B F47W mutant *in vivo* accumulate inordinately high levels of DNA damage (and genomic DNA strand breaks) upon exposure to combinations of UV-B, UV-A and visible light. This suggests that DNA repair mechanisms in such cells are potentially overwhelmed by the higher rates of DNA damage that occur in cells exposed to wavelengths of light present in terrestrial sunlight, when they host Trp-containing DNA packaging proteins.

In summary, in this piece of work, we used a three-pronged approach: (i) We provided bioinformatics-based observations of the absence of Trp; (ii) We provided the theoretical underpinnings and arguments supporting the need for DNA packaging proteins (such as the histone-like protein, HU, and the histones themselves) to lack Trp; (iii) We demonstrated the deleterious effects of the presence of Trp in such proteins, by showing that cells containing such proteins that are exposed to wavelengths of light which are ordinarily present in sunlight, accumulate inordinately high levels of DNA damage[9].

Subunit exchange between HU homodimers to form heterodimers is hindered by relocation of the positive charge at the molecule's N-terminus through addition of any affinity tag or protein[11]

It has been reported that when purified homodimers of HU-B, and HU-A,are mixed together, the facile occurrence of subunit exchange leads to the immediate transformation of such a mixture into a population of heterodimers, with leftover HU-B or HU-A molecules forming homodimers. In an interesting piece of work that we did recently, we reported that the facile nature of this subunit exchange is inhibited by the presence of any N-terminal 6XHis tag, or any type of fluorescent protein tag (*e.g.*, RFP, or Venus) present in fusion, at the N-terminus of either HU-A or HU-B. These extensions appear to cause homodimers of the two HU isoforms to remain homodimers even upon mixing, (a) with no evidence of subunit exchange, and (b) with ample evidence of the homodimers being inordinately stable. Thus, we effectively showed that the displacement of the positive charge that is naturally present at the N-terminus of HU, through the addition of any string of amino acids (affinity tags, or fluorescent proteins) happens to strengthen a hydrophobic interaction that occurs between stacked beta sheet structures at the core of the homodimer. This prevents subunit dissociation even after substantial subunit unfolding is allowed to occur in the presence of denaturants, due to the lack of the positive charge at the N-terminus (at a particular geometric location, in respect of the stacked beta sheets)[11].

What was especially interesting to us about these findings was the fact that the blocking of scope for subunit exchange between HU-A/HU-A and HU-B/HU-B homodimers does no, in any obvious manner, affect the functioning of HU as a DNA-binding protein. We connect this to our observations regarding the

differential behaviour of HU-A and HU-B in terms of the phase separation with DNA that we have discovered and also published (see above), which indicate that the two forms of HU potentially enable DNA to remain less compact in actively dividing cells that are benefiting from nutrient-rich environments, *e.g.*, during multiplication/colonization of the human gut by *E. coli*, and more compact in resting cells; however, with this being merely an enabling mechanism and not a mandatory requirement for cells.

Building a simulacrum of HU-B/A heterodimers, through the genetic fusion of HU-B and HU-A[12]

Studying the properties of natural heterodimeric HU is difficult because the purification of heterodimeric HU in large amounts away from homodimeric HU is difficult. To work around this problem, we created a genetic fusion of HU-A and HU-B, as a simulacrum of the HU-A/HU-B heterodimer, in which the polypeptide stretches corresponding to HU-A and HU-B were designed to fold and assemble with each other within the same large polypeptide, with the proximity of the two HU isoforms ensuring their preferential mutual assembly over any assembly with other HU chains. To make this simulacrum, we employed a flexible Glycine-Serine linker that was long enough (11 residues long) to not interfere with the folding and assembly of the HU-B and HU-A chain sections. Also, HU-B was placed ahead of HU-A in the chain, towards the N-terminal side of the fusion. The fusion protein folded and formed a perfect stable, DNA binding, multimeric protein simulacrum of the HU-B/HU-A heterodimer[12].

An HU-Simulacrum: A novel protein-engineered dsDNA-binding protein inspired by HU[13]

HU contains a helical N-terminal region (NTR) of ~44 residues and a C-terminal region (CTR) of ~44 amino acids. It folds into an obligate dimer with an extensive network of CTR-CTR contacts. We recently fused the CTR of *E. coli* HU-A with the CTR of *Thermus thermophilus* HU, to create a single chain(non-native) simulacrum of the canonical DNA binding elements of HU, lacking the core helical segments of HU. This simulacrum, which we called HU-simul, was monomeric, and capable of binding to double-stranded DNA (dsDNA) and cruciform DNA, but not to single-stranded DNA (ssDNA), unlike HU which also binds to ssDNA[13]. We also fused HU-Simul to Pfu DNA polymerase to create a DNA polymerase with an additional DNA-binding functionality (akin to that seen in novel protein-engineered DNA polymerases like Phusion by Invitrogen) to generate a mimic of the Phusion polymerase with the ability to perform longer amplifications (up to 10 KB).

The use of HU to detect the leaky expression of proteins[14]

Physical-biochemists and structural-biochemists (and especially those who use the tools of protein engineering) are often vexed by problems arising from the leaky expression of genes that have been placed under promoters that are believed to be capable of tightly controlling gene expression, allowing expression to occur only when an inducer is available in the medium, but found to allow apparently allow a certain amount of leaky expression to occur despite the lack of inducer. One such system is the pQE plasmid series and the M15-pRep4 *E. coli* strain. This system is believed to control gene expression very tightly. The host overexpresses the lac repressor from an additional plasmid (pRep4) to keep all copies of the gene-of-interest silenced upon the plasmid (pQE). A second such system is the pET plasmid series and the BL21 (DE3) Star pLysS *E. coli* strain. This system is also believed to very tightly control gene expression. In this system, the induction of a T7 RNA polymerase enables expression from the gene-of-interest placed under a T7 promoter upon the plasmid (pET).

When red fluorescent protein (RFP) is fused to the N-terminus of HU-A to create the fusion protein RFP-HU-A, and when the gene encoding this fusion protein is placed upon a pQE-30 plasmid transformed into an M15-pRep4 *E. coli* cell, we found that fluorescence microscopy shows varying levels of low-level expression of RFP-HU-A, detected even in the absence of any added inducer. The reason for the detection of such low-level (leaky) expression was primarily that the HU-A component was DNA-binding in character, in that this allowed the RFP-HU-A fusion to become titrated onto the nucleoid (*i.e.*, to become locally concentrated within a smaller volume inside the cell) instead of remaining diffused and spread-out over the *E. coli* cell's cytoplasm, with this facilitating a very definitive detection of its expression, folding and function[14]. Notably, we also carried out the same studies with the pET plasmid and the BL21 strain, using the Venus fluorescent protein, and HU-B, and found that this system too displays some leaky expression (although this latter piece of work remains unpublished). This work from our group demonstrates the usability of HU fluorescent protein fusion systems

to detect leaky expression by (a) exploiting the DNA binding character of HU to concentrate the expressed protein upon the nucleoid and (b) exploiting the fluorescent character of RFP to detect leaky expression sensitively through microscopy.

Ongoing research involving HU

In ongoing research in our lab, we are investigating several aspects of HU's behaviour that include (but are not limited to) (i) The impact of HU's phase separation potency on biofilm formation through HU's interactions with components other than DNA; and (ii) the interplay between HU and another nucleoid-associated protein called Dps, and the relationship between the differential phase separation behaviour of these two proteins and the transition of bacteria such as *E. coli* from the log phase of growth into the stationary phase of growth.

We believe that delving into such new aspects of HU's behaviour will prove to be crucial for the gaining of ever deeper levels of understanding of HU's role in various cellular processes that govern microbial physiology. Our findings are also likely to contribute to a broader understanding of liquid-liquid phase separation in cellular systems and its implications for bacterial biology.

Conflict of interest

All authors declare no conflict of interest.

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