

**Glutathione contributes to the adaptive
response to nitrogen starvation in
Escherichia coli.**

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Abstract

Post-transcriptional regulation underpins the post-synthesis fate of protein coding mRNA in the bacterial cell. This often involves small regulatory RNA molecules and an RNA binding protein to facilitate the interaction between a regulatory RNA molecule and its cognate target mRNA molecule. Hfq is an RNA binding protein present in diverse bacterial lineages that contributes to many different aspects of RNA metabolism during gene expression. Recent work from our group and others have discovered that individual Hfq molecules in stressed *E. coli* cells undergo liquid-liquid phase separation and form in foci like structures. Using nitrogen (N) starvation as a model stress condition, we have previously shown through super resolution microscopy that Hfq foci formation in N starved *E. coli* occurs independently of gene expression. Although the biological function of Hfq foci is still being investigated, the molecular trigger(s) that precipitates Hfq foci formation is unknown. Here, we provide new data showing a role for one of the most abundant cellular tri-peptide, glutathione, in precipitating Hfq molecules to assemble into foci. The results imply a novel facet underpinning the complexity of RNA regulation in stressed bacteria.

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Declaration of Originality

I declare that this thesis titled 'Glutathione contributes to the adaptive response to nitrogen starvation in *E. coli*.' contains my own work submitted for the degree of Doctor of Philosophy. All work performed by anyone other than myself has been acknowledged and credited. Resources and ideas from the work of others have been appropriately referenced.

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List of Abbreviations

AKG	Alpha ketoglutarate
AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
C-	20 minutes after CRO
C	Carbon
C- X	X hour after CRO
C+	Carbon replete
CFU	Colony forming unit
CRO	Carbon run out
D*	Apparent diffusion coefficient
DNA	Deoxyribonucleic acid
GCS	γ -glutamyl-cysteine synthetase
GDH	Glutamate dehydrogenase
GGT	γ -glutamyl transpeptidase
GlnB	Nitrogen regulatory protein PII-1
GlnD	Protein-PII uridylyl transferase
GlnG	DNA-binding transcriptional dual regulator NtrC
GlnK	Nitrogen regulatory protein PII-2
GlnL	Protein histidine kinase NtrB
GOR	Glutathione reductase
GS	Glutathione synthetase
GS-GOGAT	Glutamine synthetase-glutamate synthase pathway
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
H ₂ O ₂	Hydrogen peroxide
HAF	Host acquisition factor
Hfq	Host factor for RNA phage Q β replication
HOCl	Hypochlorous acid
IDD	Intrinsically disordered domain
K ⁺	Potassium
KO	Knock out
LLPS	Liquid-liquid phase separation
MG	Methylglyoxal
mRNA	Messenger RNA
MS-LCMS	Liquid chromatography mass spectrometry

N	Nitrogen
N-	20 minutes after NRO
N- X	X hour after NRO
N+	Nitrogen replete conditions
ND	Nitrogen depleted conditions
NH ₄ Cl	Ammonium chloride
NRO	Nitrogen runout
Ntr response	Nitrogen regulation stress response
O ₂ ^{•-}	Superoxide anions
OH [•]	Hydrogen peroxide
OxyR	DNA-binding transcriptional dual regulator OxyR
PALM	Photoactivated localisation microscopy
ppGpp	Guanosine tetraphosphate
pppGpp	Guanosine tetraphosphate
PTM	Post translational modification
RNA	Ribonucleic acid
RNAP	RNA polymerase
Rnase E	Ribonuclease E
RNP	ribonucleoproteins
ROOH	Hydroperoxides
ROS	Reactive oxygen species
RPc	RNA polymerase closed complex
Rpo	RNA polymerase open complex
RpoC	RNA polymerase subunit β
RpoD	RNA polymerase sigma factor RpoD (σ70)
RpoS	RNA polymerase sigma factor RpoS (σ38)
sRNA	Small RNA
TCA	Citric acid/Krebs cycle
UPEC	Uropathogenic <i>E. coli</i>
w/v	Weight per volume
WT	Wild type

1. Chapter 1: Introduction

The environmental conditions where bacteria exist are often dynamic and unpredictable. Like other organisms, it is crucial for bacteria to adapt to changes in the environment to survive and reproduce. Bacteria must adapt to shifts in pH, temperature, nutrient and water availability, amongst other environmental factors. To achieve this, bacteria have evolved a wide variety of adaptive responses. These responses can cause global changes in gene expression and metabolism, or very targeted responses, such as the upregulation of specific transporters or proteins. These responses can repair damage, halt growth and scavenge nutrients to maintain homeostasis of the cell.

Bacteria often exist in nutrient depleted environments which attenuate their growth. How they cope with this stress is not only relevant to bacterial survival, but to health and disease in humans.

1.1. Bacterial stress responses

Stress responses allow bacteria to adapt their physiology to survive in challenging environments. These responses can be very specific to one type of stress, or broad, yielding global changes which offer cross protection against multiple stresses. When *E. coli* experience environmental stress such as nutrient deprivation, high pH or oxidative stress, various regulatory and signalling pathways sense and respond to these stresses. This signalling can lead to broad transcriptional reprogramming, which allows the bacteria to adapt to the changing environment. In *E. coli*, sigma factors dictate promoter recognition and direct RNA polymerase (RNAP) to transcribe distinct sets of genes, depending on stress condition.

RpoD (also known as σ^{70} , the housekeeping sigma factor) is the major sigma factor in *E. coli* and is required for the majority of transcription during bacterial growth ^{12, 13}. There are six alternative sigma factors (FecI, RpoE, RpoF, RpoH, RpoS and RpoN), which promote transcription of different sets of genes under specific conditions, such as N limitation (RpoN) ¹⁴, heat shock (RpoH) ¹⁵, or cell dehydration (RpoS) ¹⁶.

1.1.1. General stress responses in bacteria

In *E. coli* the general stress response is reliant on the alternative sigma factor RpoS (σ^{38}) ¹⁷. Initially identified as a sigma factor which regulated a small amount of stress response genes, RpoS is now considered to be a significant multifaceted control system which regulates many diverse processes. RpoS is known to modulate responses which decrease cellular metabolism, by regulating genes involved in sugar and polyamine metabolism as well as nucleic acid synthesis and turnover ¹⁸. RpoS belongs to the same family of sigma factors as

RpoD, with both displaying preference for the same consensus promotor elements ⁷. Some promoters of genes involved in the general stress response are RpoS specific, whereas some are under the control of both RpoS and RpoD ¹⁷.

During exponential growth, RpoS expression is tightly regulated through inhibition of both transcription and translation. This regulation prevents competition for core RNAP by RpoS when cells are not experiencing stress and the general stress response is not required. Any RpoS that is made is quickly degraded. This ensures that RpoS levels remain low during growth, only increasing upon entry into stationary phase or during stress conditions ¹⁷. When cells experience stresses such as high or low pH, temperature changes, carbon starvation, low osmolarity or DNA damage, RpoS levels rapidly increase ^{19 17, 20}. During stress, RpoS associates with the RNAP holoenzyme. This complex can then recognise RpoS dependent promoter regions, leading to transcription of RpoS dependent genes. This means up to 10% of the *E. coli* genome is under direct or indirect regulation by RpoS ²¹.

Genes expressed during general stress responses encode proteins which enable the cell to cope with various aspects of stress. These include chaperones for protein refolding, thiols to reduce reactive oxygen species and enzymes to repair damage to nucleic acids. Collectively, these changes allow the cell to become adapted to the stressful environment. Although the RpoS mediated stress response can be induced by a single trigger, RpoS response is sufficiently broad and complex to convey cross protection against further secondary stresses.

One of the most characterised general stress responses is the stringent response, which occurs in response to stresses including amino acid starvation, heat shock and iron limitation. The stringent response is regulated by the small nucleotide alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate ²², produced by RelA and SpoT ²². These alarmones have wide ranging targets, and do not seem to have a binding consensus on target proteins. The binding of ppGpp alarmones to enzymes causes activation or inhibition of enzymatic processes, which can facilitate changes in processes such as DNA and nucleotide synthesis, ribosome maturation, lipid metabolism and transcription ^{22, 23}. The stringent response affects all resource consuming processes including transcription, translation and replication, and mediates the reallocation of resources from growth to amino acid synthesis ²⁴. General stress responses such as these have also been shown to be coupled to more specific stress responses, such as the nitrogen (N) stress response.

1.1.2. The nitrogen stress response

The response to N starvation is an example of a specific stress response in *E. coli*. The N regulation stress response is primarily an adaptive scavenging response, enabling bacteria to adapt to N limiting conditions ²⁵. N is assimilated via the glutamate dehydrogenase (GDH) and

glutamine synthetase-glutamate synthase pathways⁵ (Figure 1.1)^{5 26}. The GDH pathway consists of a single enzyme, glutamate dehydrogenase, which catalyses the reductive amination of α -ketoglutarate (AKG), a key product of the Krebs cycle, producing one net glutamate²⁶ (Figure 1.1 A). This reaction does not require ATP. In the GS-GOGAT pathway, the enzyme glutamine synthetase⁵ catalyses the amidation of glutamate to glutamine with the cost of one ATP, using ammonium as the N source⁵. Glutamate synthase (GOGAT) catalyses the transfer of the glutamine amide group to AKG, forming two molecules of glutamate (Figure 1.1 B). During N limitation, GS specific activity and GS expression are increased²⁵. AKG and glutamine levels are a reliable metabolic indicator of the intracellular N status of the cell, as AKG decreases and glutamine increases during N assimilation.

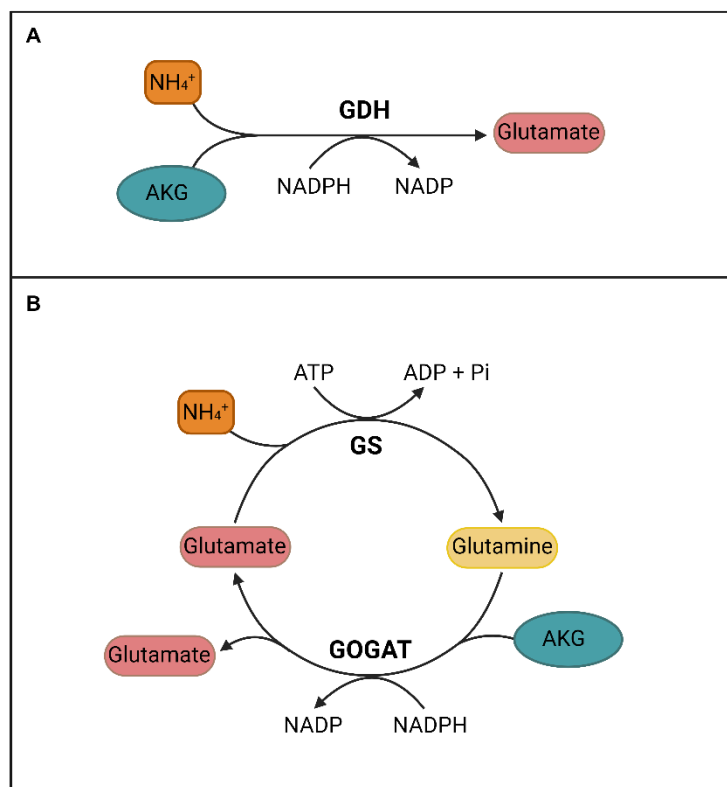


Figure 1.1: Nitrogen is assimilated via the GDH and GS-GOGAT pathways

(A) The glutamate dehydrogenase (GDH) N assimilation pathway produces one net glutamate molecule, without the use of ATP. The GS-GOGAT pathway consists of two reactions catalysed by GS and GOGAT, which catalyses the reductive amination of α -ketoglutarate using ammonium as the N source. (B) The glutamine synthetase-glutamate synthase⁵ pathway is a two-step reaction. Glutamine synthetase⁵ catalyses the reductive amination of glutamate, also using ammonium as the N source, producing one glutamine molecule and uses one ATP. Glutamate synthase (GOGAT) subsequently transfers the amide group from glutamine to α -ketoglutarate (AKG), producing two glutamate molecules⁵.

When N is abundant in the environment ammonium is readily assimilated by the GS-GOGAT pathway, producing glutamine as the intermediate ²⁶. When glutamine is present in high levels within the cytoplasm, it binds to the enzymatic regulator GlnD. GlnD has both uridylyl transferase and de-uridylylation activity, and is constitutively expressed enabling the cell to sense N quickly under all growth conditions. Glutamine binds to GlnD at one site, which controls both activities ²⁷. When bound to glutamine, GlnD removes uridine monophosphate from two N regulatory proteins; GlnB and GlnK ²⁷. De-uridylylated GlnK prevents uptake of ammonium through binding to the ammonium transporter AmtB. De-uridylylated GlnB subsequently binds to GlnL, which enhances GlnL phosphatase activity. GlnL dephosphorylates GlnG (the nitrogen (Ntr) master regulator), preventing GlnG from binding to DNA. In conditions where N is abundant, this regulatory system represses the Ntr response.

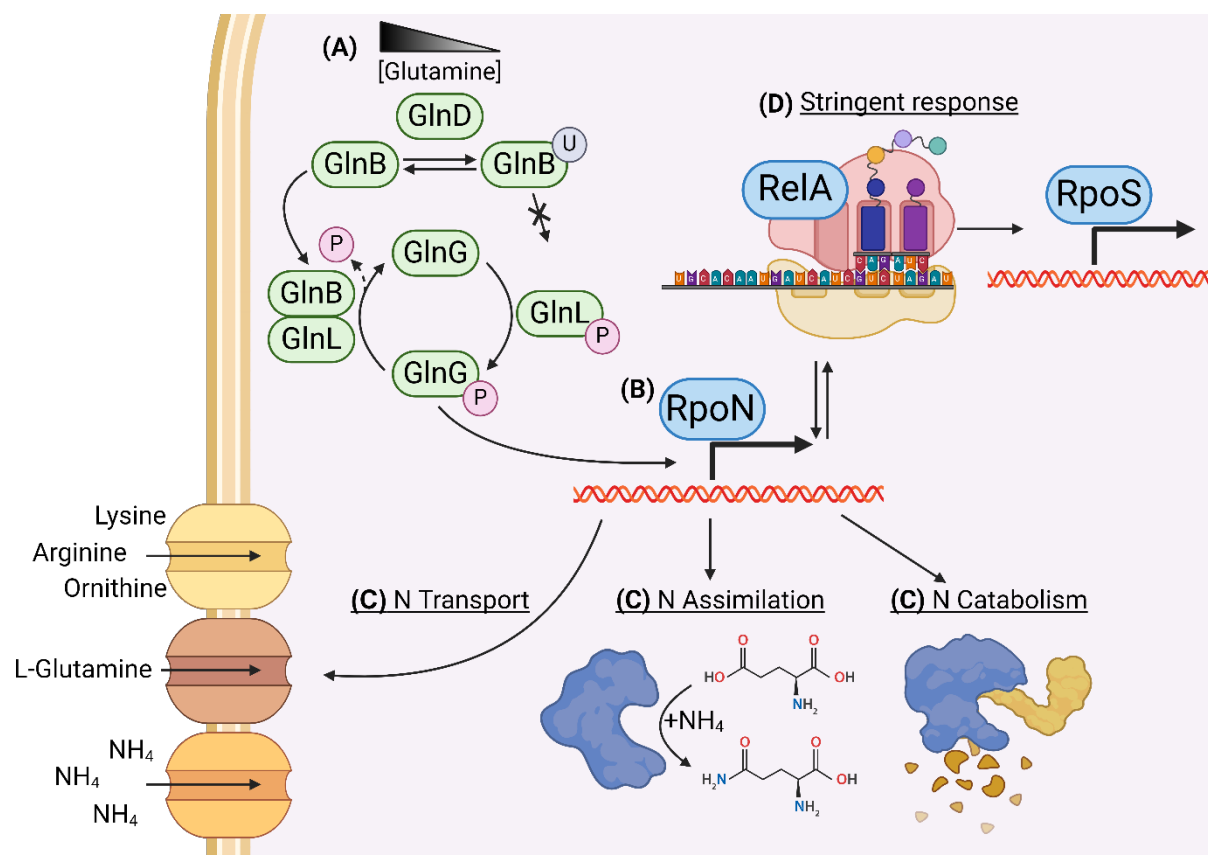


Figure 1.2: Nitrogen sensing and the nitrogen stress response in *E. coli*

(A) Low glutamine stimulates uridylyl transferase activity of unbound GlnD, which uridylylates GlnB and GlnK. Uridylylated GlnB cannot bind to GlnL, leaving GlnL open to transphosphorylation. GlnL subsequently phosphorylates GlnG, which becomes activated and binds upstream of RpoN regulated genes. (B-C) RpoN bound RNAP expresses Ntr response genes. Upregulation of these genes leads to changes in N transport, assimilation and catabolism. (D) RpoN activates RelA, an enzymatic regulator of the stringent response. Activation of RelA triggers upregulation of ppGpp and pppGpp, leading to broad transcriptional reprogramming via the general stress response sigma factor, RpoS.

In *E. coli*, external N limitation is initially sensed as a decrease in concentration of internal glutamine. When external N is sparse, intracellular glutamine depletes and therefore is not available to bind to GlnD (Figure 1.2 A). In this unbound state, GlnD uridylylates both GlnK and GlnB. Uridylylated GlnK cannot bind AmtB, allowing the continued uptake of ammonium. Uridylylated GlnB is unable to bind GlnL, GlnL undergoes transphosphorylation and subsequently phosphorylates GlnG. GlnG is a bacterial enhancer binding protein, which when phosphorylated, binds to enhancer like elements upstream of a number of RpoN-dependent promoters. Here, GlnG catalyses formation open complex formation by the RpoN RNAP holoenzyme²⁸ (Figure 1.2 B). The sigma factor RpoN is a master regulator of transcription in *E. coli*, controlling regulation of up to 2% of the genome^{29,30}. Ntr-activated genes include those involved in N assimilation, transport and catabolism (Figure 1.2 C)²⁵. The Ntr response causes upregulation of *glnA*, the gene encoding glutamine synthetase⁵, part of the GS-GOGAT pathway responsible for assimilating N²⁶. Multiple transport systems are upregulated to transport N into starved cell, including *glnHPQ* for L-glutamine and *argT* for arginine and lysine²⁹. Catabolism of N containing compounds is mediated by several operons, including *astCADBE* and *rutABCDEFG* which encode genes involved in arginine catabolism and pyrimidine degradation respectively^{31,32}.

The stringent and N stress responses in *E. coli* are linked via the enzyme RelA³³. During N starvation, *E. coli* rapidly synthesises ppGpp, the alarmone of the bacterial stringent response³⁴. As discussed above, two enzymes modulate levels of ppGpp in *E. coli*; RelA and SpoT³⁵. During N starvation, GlnG has been shown to bind upstream of *relA* in *E. coli*, thereby activating *relA* transcription³³. Upregulated RelA goes on to synthesise ppGpp and pppGpp, leading to broad changes in cellular metabolism and gene expression³⁴. This occurs through a wide range of mechanisms, including disassociation of RNAP from RpoD dependent promoters. This dissociation of RpoD leads to increased binding of other alternative sigma factor (RpoN and RpoS) and broad transcriptional reprogramming via RpoS²⁴ (Figure 1.2 D).

1.2. The role of nitrogen in bacterial physiology and pathogenesis

N is essential for all forms of life. In bacteria, it is required for biosynthesis of most major cell components, such as amino acids, proteins, nucleic acids and the cell wall. N is indirectly involved in many processes which affect the metabolic state of the cell, including the citric acid cycle and electric transport chain, which lead to the production of ATP.

N limitation is a threat to bacteria in human health and disease. Pathogenic bacteria often exist in host tissues with limited access to N, and bacteria must adapt to these conditions to establish infection. Therefore, an understanding of the N stress response is essential to

understand bacterial pathogenesis. It has been shown that N is limited in the mammalian gut, and this has implications on composition of the gut microbiota, inevitably affecting the resources available to invading pathogens^{36 37}. Bacterial pathogens such as Uropathogenic *E. coli* experience N limitation in the urinary tract³⁸, and have been found to display genetic markers of the adaptive response to N starvation³⁸. Despite its metabolite rich composition, urine is considered a relatively nutrient poor growth medium when compared to the intestinal environment³⁹. Uropathogens must therefore adapt to nutrient changes when transitioning from the intestine to the urinary tract³⁸; examples of adaptation are the production of virulence factors such as transport pumps, and the formation of biofilms^{40 3}. In some cases, bacteria respond to nutrient deficiency by forming persisters⁴¹. Both biofilms and persisters are problematic in the clinic, as these populations of bacteria display enhanced survival following exposure to antibiotics⁴⁰.

Pathogens compete with immune cells as part of the battle of immune response and evasion. The host immune response to infection can result in extensive changes to the microenvironment⁴². The host immune system changes the availability of nutrients through host sequestration of essential metals and other nutrients^{43, 44}, recruits phagocytic cells which increase nitrosative and oxidative stress⁴⁵, enhances osmolarity⁴⁶ and alters pH through acidification of the macrophage phagosome⁴⁷.

Many bacterial species have evolved complex adaptive processes to survive N limitation and starvation. As such, N starvation serves as a reasonable model to study bacteria in a state of growth attenuation. Understanding how bacteria adapt to N starvation helps us understand not just bacterial physiology and virulence but provides us with insights into new targets for therapeutic intervention.

1.3. Spatial organisation in bacteria and its role during stress

Biological organisation occurs across a multitude of scales. Traditionally, bacteria were considered unorganised compared to mammalian cells, due to their lack of classical membrane bound organelles. The lack of traditional organelles in bacteria leads us to question how the tightly packed cellular space is organised, and how bacteria achieve spatiotemporal control. Both eukaryotic and prokaryotic cells contain what are referred to as membraneless organelles, many of which form through liquid-liquid phase separation (LLPS).

1.3.1. Liquid-liquid phase separation

LLPS is the separation of a homogenous mixture into two distinct phases via spontaneous de-mixing. These phases are commonly referred to as the dense and dilute phase (Figure 1.3)

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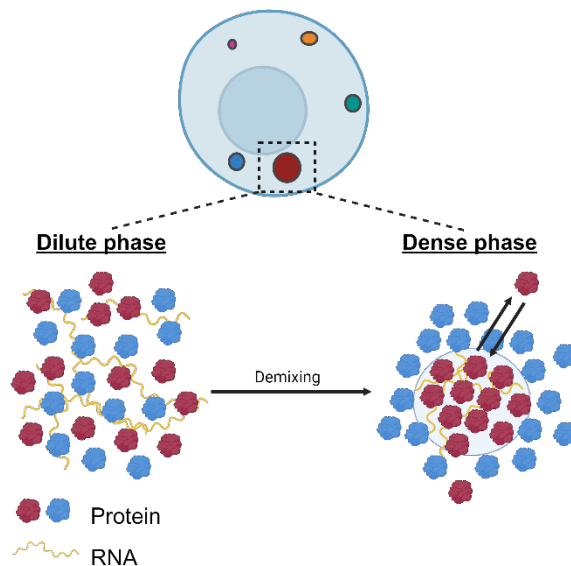


Figure 1.3: Liquid-liquid phase separation of proteins

Liquid-liquid phase separated proteins spontaneously de-mix from a dilute phase containing a mix of proteins and other cellular components such as RNA (left) to a dense (right) phase.

The separation of molecules by LLPS relies mainly on multiple non-covalent interactions between molecules, for example Van der Waals forces, electrostatic and hydrophobic interactions as well as hydrogen bonding ^{49, 50}. These forces cause some molecules to cluster together in a more energetically favourable dense structure, whilst others remain in the dilute phase. Once a threshold critical concentration is met, referred to as the 'spinodal' point, phase separation occurs ⁵⁰.

The propensity of molecules to liquid separate depends on their structure and interactions with other components of the mixture, as well as environmental factors such as pH, temperature, protein concentration and osmotic pressure ⁴⁸. The structure of proteins determines the number and type of interactions it can have with other components of the cell, and often biomolecular condensates are enriched with multivalent molecules ^{50 51}. The more available places a molecule can interact the more likely it is to be able to phase separate. It is common for proteins to require interactions with other polymers, such as RNA and DNA, to phase separate ^{50, 52}. RNA is a key component of many LLPS proteins structures ⁵³. Interestingly, the

length, structure, sequence and concentration of RNA all effect dynamics of LLPS ^{52, 54 55}. The secondary structure of mRNA determines whether it can self-associate, and be recruited or excluded from LLPS compartments ⁵⁶.

Many liquid phase separated proteins contain an intrinsically disordered domains (IDDs) ^{57 58}, which may fluctuate between different confirmations. Fluctuation between confirmations occurs as IDD typically do not fold to form stable folded structures, giving them dynamic qualities ⁵⁹. IDDs typically contain a high percentage of charged amino acids, such as arginine (R), aspartic acid (D), histadine (H), lysine (K) and glutamic acid (E) ^{59 60}. The electrostatic forces created by these charged amino acids is thought to play an important role in governing LLPS of proteins. These IDDs play a significant role in LLPS of many, but not all proteins.

Post translational modification (PTM) of proteins plays an important role in regulating LLPS of proteins. PTMs can change the hydrophobicity, structure and charge of proteins involved in LLPS, thereby changing affecting their phase transition behaviour. Common PTMs include phosphorylation, glycosylation, methylation, Ubiquitination, S-Nitrosylation, N-Acetylation and carbonylation (of ketones & aldehydes). Some PTMs have been shown to occur in response to particular stresses, such as S-Nitrosylation and nitration which occur during nitrosative and oxidative stress respectively, and these PTMs can act as major signalling mechanisms under these conditions ⁶¹.

Phosphorylation is one of the most common forms of PTM. It acts as a quick way to regulate protein properties in response to various signals, and provides extensive regulation of enzyme activity in *E. coli* ⁶². Phosphorylation is catalysed by protein kinases which create covalent bonds between phosphate group of ATP and the hydroxyl groups on amino acids ⁶³. As phosphate groups are negatively charged, phosphorylation introduces a negative charge to proteins, altering their electrostatic charge and therefore their interactions with other molecules along the polypeptide backbone⁶². Phosphorylation can both enhance and inhibit LLPS, and has shown to be highly specific to the target protein ⁶⁴⁻⁶⁶. Similarly, acetylation also changes the charge of proteins, changing positively charged residues to neutral – this has been shown to regulate stress granule formation *in vivo* ⁵⁷.

Another way in which PTMs can influence LLPS is by changing the hydrogen bonding capacity of a protein, rather than by altering the net charge. An example of this is arginine methylation, which increases hydrophobicity thereby changing the behaviour of the protein ⁶⁷. Ubiquitination is unique compared to the PTMs mentioned previously, in that it can bring about introduction of new multivalent interactions and polyubiquitin chains are known to interact with various recognition motifs, however this PTM does not occur in *E. coli* ⁶⁸. Many these PTMs,

electrostatic forces and non-covalent interactions work synergistically to govern LLPS dynamics ^{2, 69}.

Liquid-liquid phase separation is a common way for non-membrane bound structures to form ⁷⁰. Whilst there is no definitive test to determine if a biological condensate forms via LLPS, Hyamen *et. al*/described a set of characteristics that help us define the liquid like properties of these structures ⁴⁹ (Figure 1.4). LLPS structures are nucleated to form spherical shapes due to surface tension, with smaller droplets fusing to form larger structures, a process which is reversible. Molecules within the dense phase are able to move in and out into the dilute phase and vice versa, diffusing across the boundary ⁴⁹. LLPS can also be dependent on concentration, with increased protein concentration accelerating de-mixing ⁷¹.

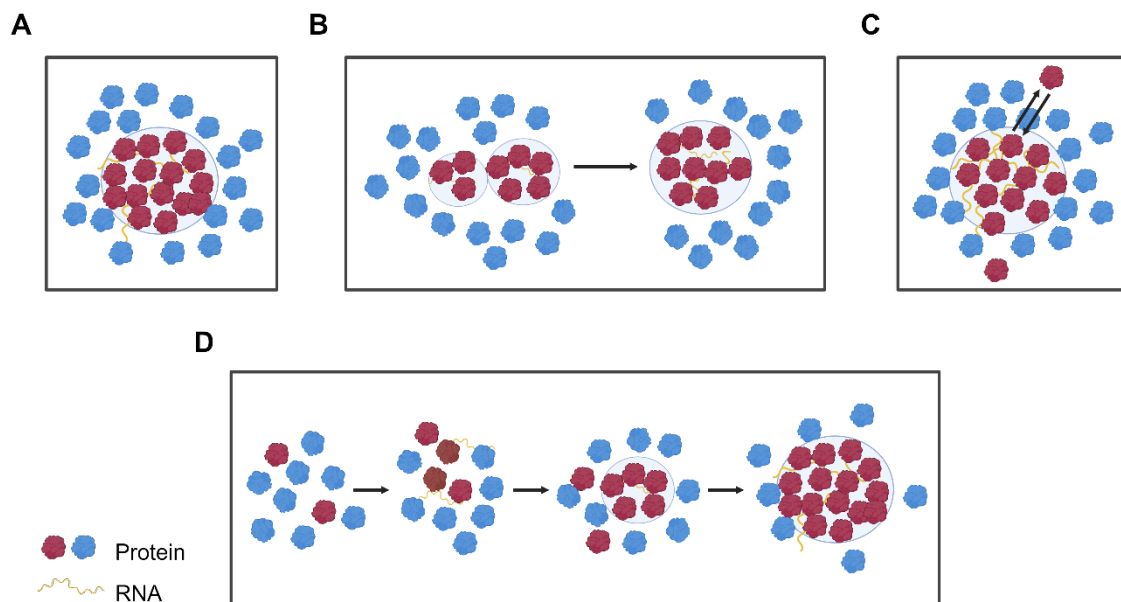


Figure 1.4: Characteristics of liquid-liquid phase separated proteins

(A) LLPS structures form spherical structures (B) Smaller LLPS protein droplets fuse together to form larger structures (C) Individual proteins can move in and out of the LLPS structure (D) LLPS structures are concentration dependent.

The formation of condensed phases during LLPS are often reversible, however LLPS proteins can form gels when components of the dense phase cross link with each other and become physical gels ^{72 73}. These gels often require high salt or denaturation to dissolve ⁷⁴. Aggregation is said to have occurred when irreversible molecular interactions are formed and proteins enter

a higher ordered state, which is irreversible. The progression of LLPS structures from liquid to solid states has implications in disease pathology such as in ALS or Alzheimer's, which are caused by LLPS protein plaques ^{75, 76}.

1.3.2. Liquid-liquid phase separated structures in biology

Examples of non-membrane bound structures are becoming increasingly common in biology. These structures ordinarily form in response to some sort of stimulus and consist of a variety of proteins and nucleic acids. Collectively for simplicity, these non-membrane bound structures are commonly referred to as biomolecular condensates, independently of known composition or mechanism of formation.

The nucleolus was one of the first biomolecular condensates to be discovered in the 1830s ⁷⁷. Since then, a vast array of biomolecular condensates have been identified in a range of eukaryotic species, including Cajal bodies ⁷⁸, germ granules ⁷⁹, P bodies and stress granules ^{75, 80, 81}. These structures have been extensively studied in eukaryotic cells, and whilst some functions have been suggested, we still have little conclusive evidence about what most of these structures do. It has been proposed that stress granules may sequester viral RNA ^{82, 83}, that P granules are involved in mRNA turnover ^{75, 80, 81} and Cajal bodies have been implicated in RNA-related metabolism ⁷⁸.

In prokaryotes, size has historically been a limiting factor in studying such biomolecular condensates, however recent advancements in technology have facilitated the discovery of biomolecular condensates in bacteria. These biomolecular condensates include BR bodies ⁸⁴, RNAP clusters ⁸⁵, Polyphosphate granules ⁸⁶, ParB-ParS clusters ⁸⁷, carboxysomes ⁸⁸ and Hfq foci ⁸⁹. These structures have very diverse physical properties, locations and compositions and stability can last from minutes to days ^{90 91 89, 92}. Formation occurs upon exposure to a multitude of stimuli ^{50, 52, 89, 93}. Recently, LLPS has been proposed as a driving force of many of these biomolecular condensates ^{50, 52, 89 70}.

Whilst the functions and assembly mechanisms of most of these biomolecular condensates have been suggested, many of these proposed functions have not been conclusively demonstrated, and further work must be carried out to confirm most models. It has been hypothesised that functions can include localising reactions by concentrating or segregating components, thereby accelerating or slowing down reactions.

1.3.2.1. BR Bodies in *Caulobacter crescentus*

The best characterised of these bacterial biomolecular condensates are bacterial ribonucleoprotein bodies (BR bodies). BR bodies were the first biomolecular condensate shown to form in bacteria via LLPS, in *Caulobacter crescentus* ⁸⁴.

BR bodies form in response to stress conditions, becoming highly enriched in response to heat shock and ethanol addition, as well as exposure to ROS, cold shock and carbon starvation albeit to a lesser extent ⁸⁴.

BR bodies have liquid-like properties and have been shown to form spherical droplets *in vitro* ⁸⁴. They are composed of RNase E and the RNase E degradosome components; the RNA helicase RhlB, exonucleases RNase D and PNPase, as well as the TCA cycle related metabolic enzyme aconitase, and various species of unstructured RNA and sRNAs, generally excluding structured RNAs such as tRNA and rRNA ^{84, 94}. RNase E requires RNA to assemble BR bodies and recruit other elements to it, and disassembly occurs upon relief of stress. LLPS of BR bodies are dependent on the RNase E C-terminal domain, the intrinsically disordered domain at the C terminus of the protein which contains both RNA binding sites, protein binding sites and membrane insertion domain, which is used as a scaffold ⁸⁴. BR bodies have been shown to reshape the mRNA pool of bacteria by stimulating the rate of decay of this RNA species ⁹⁵.

1.3.2.2. RNAP clusters in *E. coli*

RNA polymerase (RNAP) forms foci in exponentially growing *E. coli* ⁹⁶. This clustering has been linked to nucleoid localisation and implicated in RNAPs role in transcription ⁹⁷. It has recently been proposed that two types of RNAP exist within these clusters; actively transcribing, and non-transcribing ⁸⁵. It has been suggested that ribosomal RNA (rRNA) transcription rates may be accelerated due to an increase in re-initiation rates, caused by the confinement of non-transcribing RNAP to clusters. NusA, the antitermination factor, has been proposed as the nucleating factor of RNAP clusters, as it forms droplets both *in vivo* and *in vitro* ⁸⁵. These clusters have been proposed to form by LLPS as they display some of the hallmarks of LLPS (Figure 1.3), RpoC (a component of RNAP) has been observed moving in and out of the biomolecular condensates ⁸⁵. RNAP clusters also disperse upon addition of 1-6 hexanediol, an aliphatic alcohol which is known to disperse LLPS structures by disrupting weak hydrophobic interactions ^{85, 98}. Our laboratory has also observed these RNAP structures (unpublished).

1.3.2.3. Hfq foci in *E. coli*

Hfq foci are a recently discovered LLPS separated structure identified by our group during N starvation ^{89, 99}.

1.3.2.3.1. The RNA chaperone protein Hfq

Hfq is a hexameric sm-like protein (Figure 1.5) approximately 65Å in diameter, which is highly conserved across bacterial species and involved in multiple aspects of RNA metabolism ^{100, 101 102, 103}.

Hfq was first discovered in *E. coli* as a host factor for bacteriophage Q β essential for the phage replication ¹⁰⁴. Hfq has since been shown to be a key RNA binding protein with a wide range of functions which contribute to post-transcriptional regulation ¹⁰⁵. Hfq co-precipitates with many species of RNA, the most common being small RNA (sRNA) and mRNA ^{106, 107}. This is unsurprising as the most common physiological roles of Hfq are generally dependent on interactions with regulatory sRNAs and facilitating binding to their mRNA targets ^{105, 108}. This results in regulation of translation and degradation of RNA ¹⁰⁵. Hfq is essential for the translation of RpoS and RpoE, alternative sigma factors which allow *E. coli* to respond to stress ^{109, 110}.

Hfq binds RNA at four sites, the proximal and distal faces, the rim and the C terminal region ¹⁰⁸ (Figure 1.5). Each face interacts differently with RNA, preferring specific sequence motifs ^{105, 111}. In *E. coli*, the distal face shows preferential binding to A rich oligonucleotides, whereas the proximal face binds polyU sequences ¹¹²⁻¹¹⁴. The rim of Hfq interacts with RNA in a non-specific manner via conserved residues R16, R17 and R18, which create an area of positive charge ¹¹⁵.

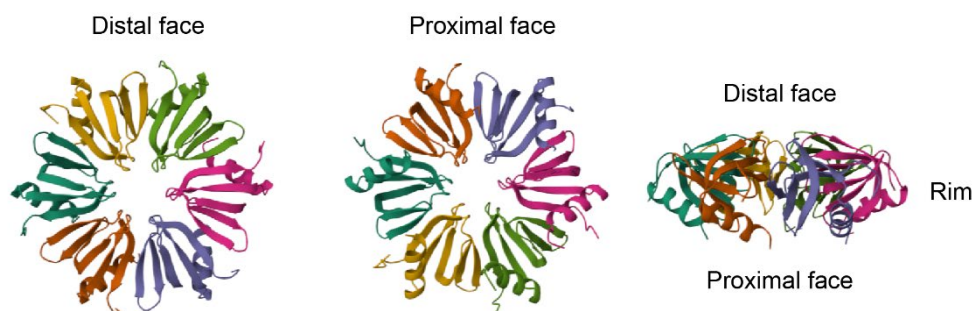


Figure 1.5: Hfq structure in *E. coli*

Cartoon representation of hexameric Hfq structure, showing the distal face, proximal face and rim.

Hfq forms extensive networks through binding multiple RNAs. RIL-seq (RNA interaction by ligation and sequencing) experiments, which allow genome-wide mapping of RNA-RNA interactions, have shown that Hfq facilitates approximately 2800 sRNA-mRNA interactions over various stages of nutrient limitation, providing us with insight into the diverse and dynamic nature of these interactions¹¹⁶. These dynamic interactions facilitate rapid exchanges of RNA, allowing Hfq to facilitate quick and specific responses to stress, despite its ability to bind diverse pairs of RNA¹⁰⁵.

In addition to facilitating base pairing between sRNA and mRNA, Hfq also plays a role in many other processes in the cell. Hfq has a role in ribosomal biogenesis and function by binding ribosomal RNA¹¹⁷, and affects translational fidelity through interactions with tRNA¹¹⁸. Hfq has also been shown to bind both linear and supercoiled double stranded DNA, influencing DNA fate¹¹⁹ as well as RNA polymerase¹²⁰.

1.3.2.3.2. Hfq foci formation in response to stress

Interestingly, Hfq has been shown to liquid-liquid phase separate in response to certain stress conditions^{52, 89}. McQuail *et al.*, first showed that Hfq forms distinct foci during N starvation in *E. coli* (Figure 1.6)⁸⁹.

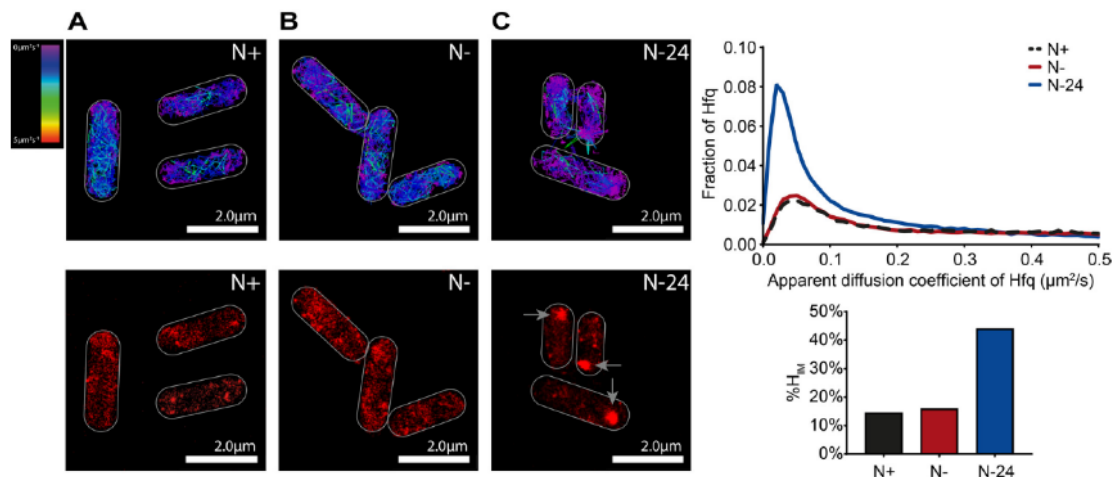


Figure 1.6: Formation of Hfq foci

When N is replete (N+) Hfq is distributed around the cell. 24 hours after N starvation ensues (N-24), Hfq forms single distinct foci. The apparent diffusion coefficient of Hfq decreases as cells progress through N starvation (top right). %H_{IM} values show the amount of immobile Hfq molecules within the sample. Hfq foci contain a higher percentage of immobile molecules and therefore a higher %H_{IM} value (bottom right). Figure from McQuail *et. al*, *JBC*, 2020.

The Hfq foci form gradually over time, independently of *de novo* transcription and translation, and disperse upon relief of N stress (Figure 1.7) ⁸⁹. Interestingly, despite being observed in response to N starvation, formation of Hfq foci is not reliant on canonical regulators known to play a role in the adaptive response to N starvation ²⁹ or general stress responses (RpoS or RelA) ⁸⁹.

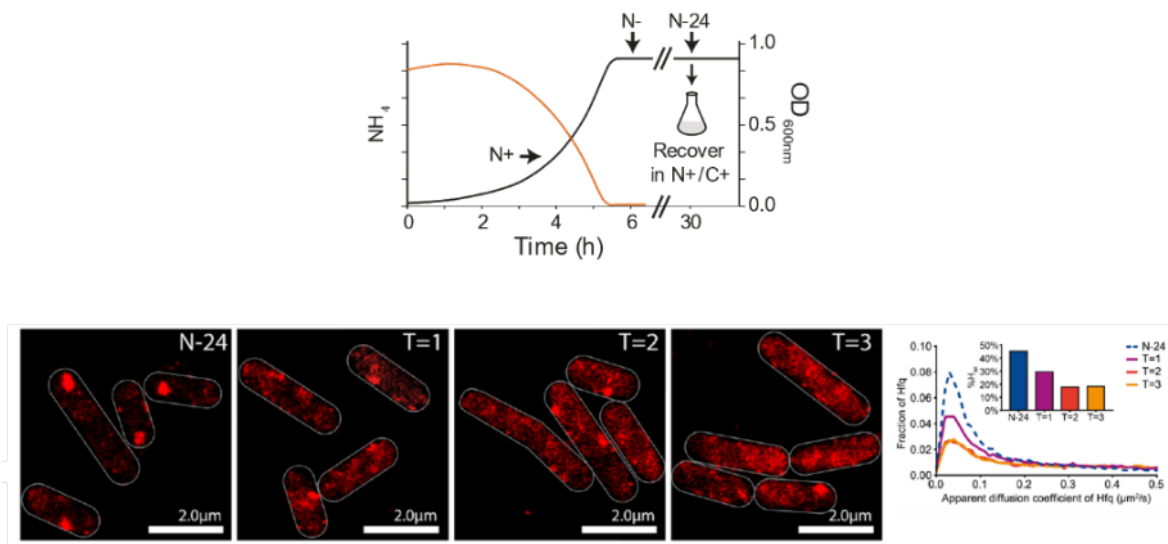


Figure 1.7: Hfq foci are reversible upon relief of stress

E. coli grown in N limiting conditions were sampled 24 hours into N starvation (N-24) and recovered in media containing N. Hfq foci dispersed upon relief of N stress. Dispersal of Hfq foci was reflected by an increase in apparent diffusion coefficient of Hfq and a decrease in the amount of immobile Hfq molecules (%H_{IM}). Figure from *McQuail et. al, JBC, 2020*.

Whilst initially seeming to be a response specifically to N starvation, data from other studies has demonstrated formation of Hfq foci in response to other stresses ⁵². In addition to N starvation, Hfq foci are also observed in late stationary phase (where glucose is not the limiting nutrient), osmotic stress and cold shock ⁵². Hfq foci do not form in response to carbon starvation (Figure 1.8) or oxidative stress ^{52, 89}. It is perhaps unsurprising that other conditions have emerged which supported Hfq foci formation, given the convergence and cross talk between different stress conditions in the cell ^{17, 33}. Similar to BR Bodies and RNAP clusters, there may be multiple seemingly independent pathways which contribute to their formation.

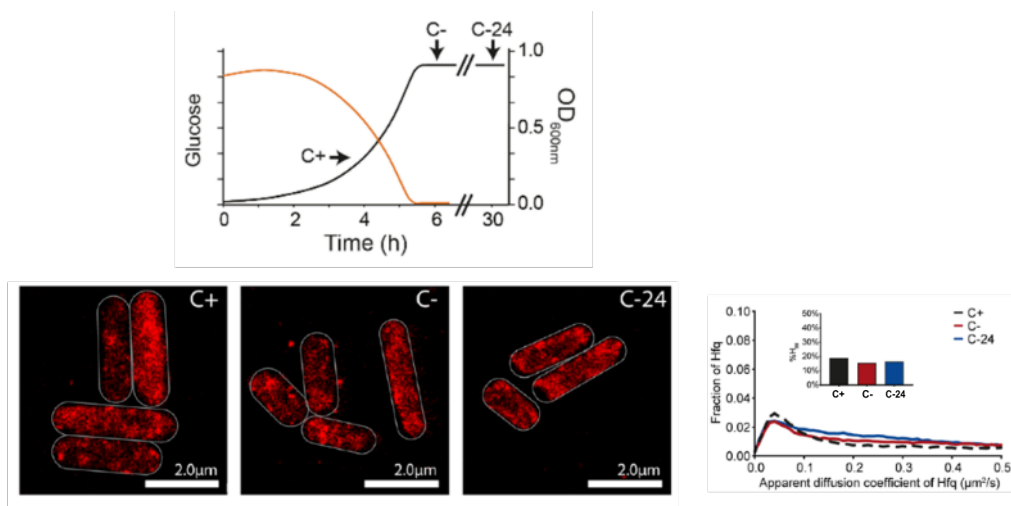


Figure 1.8: Hfq foci do not form in response to carbon starvation

When *E. coli* are grown in gutnick minimal media containing limiting glucose as the sole carbon source, Hfq foci do not form. This is reflected by the apparent diffusion coefficient and %H_{IM} of Hfq (right graph), which remain the same from exponential growth to 24 hours of carbon starvation (C-24). Figure from McQuail *et. al*, *JBC*, 2020.

Whilst the Hfq foci appear to be similar structures induced by these variety of stresses, it is currently unknown if the foci contain the same components under each stress condition.

Unlike many LLPS structures, formation of Hfq foci during N starvation does not seem to be dependent on the intrinsically disordered C-terminal domain of Hfq ⁵². They have however been shown *in vitro* to be concentration dependent and reliant on a crowding agent for formation, although *in vivo* an increase in concentration of Hfq does not seem to be the driving factor of phase separation, suggesting that phase separation *in vivo* requires factors other than an excess of Hfq ⁵². One such factor is RNA. As with many LLPS structures, RNA binding plays an important role in Hfq foci formation ^{52, 89}. Strains containing Hfq mutations at D9A (which increases RNA binding) were shown to have altered foci formation dynamics, with foci appearing denser by N-24. Y25D Hfq mutant strains (which have decreased RNA binding) displayed compromised Hfq foci when compared to wild type ⁸⁹. Further to this, *in vitro* experiments have demonstrated that Hfq foci formation is dependent on RNA binding to the distal rim of Hfq ⁵². PolyA RNA was shown *in vitro* to increase the rate of Hfq droplet formation, as did PolyU albeit to a lesser extent ⁵². It has also been shown that sRNA-mRNA pairs facilitated by Hfq binding (such as SgrS and PtsG) can LLPS with Hfq *in vitro* ⁵².

1.3.2.3.3. Composition and function of Hfq foci

The composition of the Hfq foci is currently being investigated in the field. The Hfq foci have been shown to co-localise with RNase E during long term N starvation (a structure referred to as the H-body). However, RNase E association is not a requirement for Hfq foci formation in general ⁹⁹. The essential protein RNase E is a bacterial ribonuclease, which plays a role in processing of ribosomal RNA and is a central enzyme in RNA degradation ¹²¹. The RNA degradosome consists of two RNases (RNase E and PNPase), the DEAD-box RNA helicase RhlB, and the glycolytic enzyme enolase ¹²¹. RNase E and PNPase carry out RNA degradation, whilst RhlB facilitates RNA stem loop unwinding. RNase E also acts as scaffold protein within the RNA degradosome ^{121 122}. Together, these proteins facilitate mRNA degradation in *E. coli*. Minimal components of the RNA degradosome PNPase and RhlB have been shown to associate with the Hfq foci during N starvation, and are recruited by RNase E. However, Hfq and RNase E form foci in the absence of both PNPase and RhlB, demonstrating that these proteins are not required for these phenomena ⁹⁹. In *E. coli*, Hfq bound to sRNAs interacts with the RNA degradosome, leading to targeted degradation of mRNAs ^{121, 122}. Therefore, co-localisation of Hfq and the RNA degradosome into H bodies during N starvation could suggest the foci play a role in RNA degradation ⁹⁹. Interestingly, components of the RNA degradosome have not been shown to co-localise with Hfq foci forming in long term stationary phase ⁵², suggesting differences in composition or function of Hfq foci which form in different stress conditions.

Hfq foci formation is influenced by the composition and concentration of RNA during long term stationary phase ^{52, 99}. Formation of the Hfq foci in both N starvation and long-term stationary phase has some level of requirement on RNA interaction and RNA type. Addition of PolyA RNA increases the rate of Hfq droplet formation *in vitro*. The addition of PolyU RNA also increases the rate of Hfq droplet formation, but to a lesser extent ⁵². Two RNAs known to interact with Hfq, SgrS sRNA and ptsG mRNA, co-localise within these Hfq droplets, demonstrating that RNA are present in the Hfq foci ⁵². Interestingly, the sensitivity of Hfq foci to RNA seems to differ between growth phases. When rifampicin (which inhibits transcription) is added to exponentially growing *E. coli* in LB, Hfq foci disperse following treatment, however rifampicin treatment during late stationary phase and N starvation does not disperse or affect the gradual formation of the Hfq foci ^{52, 89}. This correlates with data showing that Hfq foci are less dense during earlier stages of growth, when the metabolism is more active ⁸⁹.

Proteins can also influence the liquid phase separation of Hfq into foci. Very recently the formation of Hfq during long term stationary phase (after overnight incubation) has been shown to require TmaR, a regulator of the sugar phosphotransferase system ⁵². TmaR is known to regulate sugar metabolism in *E. coli*, by sequestration of the phosphotransferase

system protein EI¹²³. WT *E. coli* form Hfq foci during long term stationary phase, however Hfq foci are not present in $\Delta tmaR$ mutant bacteria Figure 1.9. It has also been shown that TmaR co-localise with Hfq (Figure 1.10). These data together suggest that TmaR may be a component of part of the Hfq foci which form during long term stationary phase.

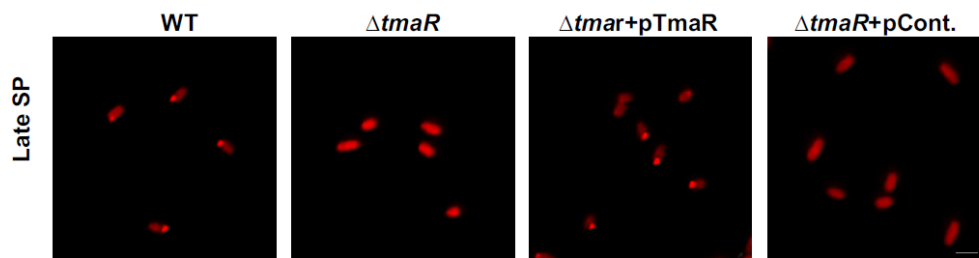


Figure 1.9: Hfq foci formation during late stationary phase requires TmaR

Images of Hfq-mCherry in live WT *E. coli* during late stationary phase². From left to right: Hfq foci form during late stationary phase in WT *E. coli*; Hfq foci do not form in the absence of TmaR ($\Delta tmaR$); Hfq foci form in $\Delta tmaR$ expressing TmaR from a plasmid (pTmaR); Hfq foci do not form in $\Delta tmaR$ containing vector only (pCont.). Scale bar 2 μ M. Figure from *Goldberger et. al, Cell Reports, 2022*.

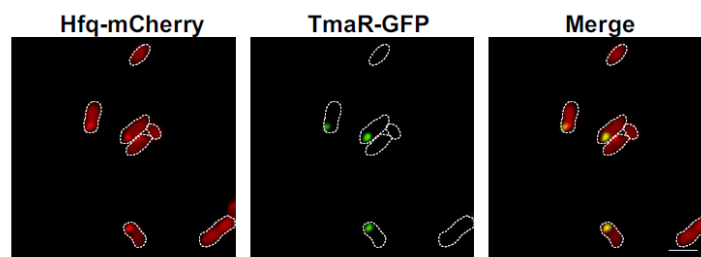


Figure 1.10: Hfq and TmaR co-localise during long term stationary phase

Images show live WT *E. coli* expressing chromosomal Hfq-mCherry and TmaR-GFP during long term stationary phase. Scale bar 2 μ M. Figure from *Goldberger et. al, Cell Reports, 2022*.

These data could suggest that the Hfq foci play a role in maintenance of RNA, spatial organisation of sRNA with mRNA or target proteins within the cell. The foci have also been linked to sugar metabolism during stationary phase, but not N starvation. As the role of TmaR is currently the subject of investigation of another group, we will not investigate this protein specifically within this thesis.

At the time of writing, the exact composition, trigger and function of the Hfq foci during both late stationary phase and N starvation is unknown. It is also unknown if the Hfq foci form via the same trigger or have the same composition in different stresses. Here we provide new data showing a role for one of the most abundant cellular tripeptides (glutathione) in precipitating Hfq molecules to assemble into foci during N starvation.

1.4. Aims

In this work we aimed to elucidate the molecular trigger for the formation of Hfq foci in *E. coli*. As discussed in the introduction, it has been established that Hfq foci form independently of transcription and translation. We therefore reasoned that formation of the Hfq foci could be triggered by metabolic changes.

Here, we aimed to explore the role of cellular metabolism on Hfq foci formation, and whether Hfq foci are linked to the survival of *E. coli* during N starvation.

2. Chapter 2: Materials and Methods

2.1. Strains and plasmids

Table 1: Strains list

Strain	Description	Source
Name		
WT MG1655	<i>E. coli</i> K-12 rph-1	<i>E. coli</i> Genetic Stock Centre
$\Delta gshAB$	MG1655 $\Delta gshAB::kan$ P	This work
$\Delta gshAB$ Hfq-pAmCherry	MG1655 Hfq-pAmCherry $\Delta gshAB::kan$ P	This work
$\Delta gshAB$ Hfq-pAmCherry (cured)	MG1655 Hfq-pAmCherry $\Delta gshAB::P$	This work
$\Delta tmaR$	MG1655 $\Delta tmaR::kan$ P	This work
$\Delta gshAB\Delta tmaR$	MG1655 $\Delta gshAB \Delta tmaR::kan$ P	This work
$\Delta gshAB\Delta tmaR$ Hfq-pAmCherry	MG1655 Hfq-pAmCherry $\Delta gshAB \Delta tmaR::kan$ P	This work
Δggt	MG1655 $\Delta ggt::kan$ P	This work
Δggt Hfq-pAmCherry	MG1655 Hfq-pAmCherry $\Delta ggt::kan$ P	This work
$\Delta gshAB\Delta ggt$	MG1655 $\Delta gshAB \Delta ggt::kan$ P	This work
$\Delta gshAB\Delta ggt$ Hfq-pAmCherry	MG1655 Hfq-pAmCherry $\Delta gshAB \Delta ggt::kan$ P	This work
rnE-pAmCherry	MG1655 rnE-pAmCherry::kan P	This work
$\Delta gshAB$ rnE-pAmCherry	MG1655 rnE-pAmCherry $\Delta gshAB::kan$ P	This work
rpoC-pAmCherry	MG1655 rpoC-pAmCherry::amp P	This work
$\Delta gshAB$ rpoC-pAmCherry	MG1655 rpoC-pAmCherry $\Delta gshAB::amp$ P	This work
Hfq-mCherry	MG1655 hfq-mCherry-kan λ	Made by Dr Josh McQuail
Hfq-pAmCherry	MG1655 hfq-pAmCherry-kan λ	Made by Dr Josh McQuail
Hfq-pAmCherry (cured)	MG1655 hfq-mCherry λ	Made by Dr Josh McQuail
JE1 (DE3)	λ (DE3 [<i>lacI lacUV5-T7p07 ind1 sam7 nin5</i>])	Stratagene
XL1 blue	<i>E. coli</i> standard cloning strain. Contains endA1 and recA1 mutant recombinase to prevent unwanted recombination.	Stratagene

Table 2: Plasmid list

Plasmid Name	Description	Insert/gene name	Antibiotic resistance	Source
pET33b	Expression vector derived from pet328a. Contains a 15bp sequence encoding protein kinase A (PKA) site, allowing for 32P protein labelling. Used for expressing recombinant proteins with N or C terminal 6xHis tags. Protein expression is under the control of a T7 promotor with a Lac operator.		Kanamycin	Novagen
pET33b Gp2	Expression vector used for expressing recombinant C terminally His tagged Gp2.	Gp2	Kanamycin	Made by Dr Andrey Shadrin
pBAD18	Used for the expression of genes from a tightly regulated araBAD promotor. Positive and negative regulation occurs by the promotor regulator araC, after the addition of arabinose or glucose respectively.		Ampicillin	¹²⁴
pBAD18 Gp2	Used for expressing recombinant C terminally His tagged Gp2.	Gp2	Ampicillin	Made by Dr Andrey Shadrin
pBAD18 Gp5.7	Used for expressing recombinant C terminally His tagged Gp5.7.	Gp5.7	Ampicillin	Made by Dr Aline Tabib-Salazar
pBAD18-ColE2-E3C	Used for expressing recombinant C terminally His tagged Colicin E2-E3C	Colicin E2-E3C	Ampicillin	This work
pBAD18 ColE2_Gp2	Used for expressing recombinant C terminally His tagged Colicin E2-Gp2	Colicin E2-Gp2	Ampicillin	This work
pBAD18 ColE2_R65E	Used for expressing recombinant C terminally His tagged Colicin E2-R56E	Colicin E2-R56E	Ampicillin	This work
pBAD18 ColE2_Gp2_V2	Used for expressing recombinant C terminally His tagged Colicin E2-Gp2 version 2	Colicin E2-Gp2-V2	Ampicillin	This work
pBAD18 ColE2_R65E_V2	Used for expressing recombinant C terminally His tagged Colicin E2-R56E version 2	Colicin E2-R56E-V2	Ampicillin	This work
pBAD18 ColE2-Gp5.7	Used for expressing recombinant C terminally His tagged Colicin E2-Gp5.7	Colicin E2-Gp5.7	Ampicillin	This work
pBAD18 ColE2-R24A	Used for expressing recombinant C terminally His tagged Colicin E2-R24A	Colicin E2-R24A	Ampicillin	This work
pBAD18 ColE2-E3C-Gp2	Used for expressing recombinant C terminally His tagged Colicin E2-E3C-Gp2	Colicin E2-E3C-Gp2	Ampicillin	This work
pBAD18 ColE2-E3C-R56E	Used for expressing recombinant C terminally His tagged Colicin E2-E3C-R56E	Colicin E2-E3C-R56E	Ampicillin	This work

pUC57 empty	Expression vector derived from pUC19. Protein expression is under the control of a T7 promotor with a Lac operator.		Kanamycin	Gifted from Syngulon
puC57-ColE2-E3C	Used for expressing recombinant C terminally His tagged Colicin E2-E3C	Colicin E2-E3C	Kanamycin	Gifted from Syngulon
pUC57 ColE2ΔC	Used for expressing recombinant C terminally His tagged Colicin E2-ΔC	Colicin E2ΔC	Kanamycin	Gifted from Syngulon
pUC57 ColE2_Gp2	Used for expressing recombinant C terminally His tagged Colicin E2-Gp2	Colicin E2-Gp2	Kanamycin	This work
pUC57 ColE2_R56E	Used for expressing recombinant C terminally His tagged Colicin E2-R56E	Colicin E2-R56E	Kanamycin	This work
pUC57 ColE2_Gp2_V2	Used for expressing recombinant C terminally His tagged Colicin E2-Gp2 version 2	Colicin E2-Gp2-V2	Kanamycin	This work
pUC57 ColE2_R56E_V2	Used for expressing recombinant C terminally His tagged Colicin E2-R56E version 2	Colicin E2-R56E-V2	Kanamycin	This work
pUC57 ColE2-Gp5.7	Used for expressing recombinant C terminally His tagged Colicin E2-Gp5.7	Colicin E2-Gp5.7	Kanamycin	This work
pUC57 ColE2-R24A	Used for expressing recombinant C terminally His tagged Colicin E2-R24A	Colicin E2-R24A	Kanamycin	This work
pUC57 ColE2-E3C-Gp2	Used for expressing recombinant C terminally His tagged Colicin E2-E3C-Gp2	Colicin E2-E3C-Gp2	Kanamycin	This work
pUC57 ColE2-E3C-R56E	Used for expressing recombinant C terminally His tagged Colicin E2-E3C-R56E	Colicin E2-E3C-R56E	Kanamycin	This work

2.2. Growth conditions

Unless otherwise stated, overnight cultures were grown in gutnick minimal media supplemented with 10 mM ammonium chloride as the sole nitrogen source, containing appropriate antibiotic for selection. Antibiotics were supplemented at 100 µg/mL ampicillin, 35 µg/mL chloramphenicol and 50 µg/mL kanamycin. Antibiotics were generally not present in day cultures unless specified. When used to inhibit transcription or translation in day cultures, antibiotics were used at 100 µg/mL for rifampicin and 150 µg/mL for chloramphenicol respectively.

Bacterial day cultures for nitrogen starvation experiments were grown in gutnick minimal media supplemented with 3 mM ammonium chloride (Table 3).

Bacterial day cultures for carbon starvation experiments were grown in gutnick minimal media supplemented with 10mM ammonium chloride and 0.06% w/v glucose.

Recovery experiments were carried out using gutnick minimal media supplemented with 10 mM ammonium chloride. For experiments containing 1-6 hexanediol (HEX), HEX was added at 10% w/v.

For internal expression of proteins during cell growth, bacteria were transformed with pBAD18 plasmids via heat shock transformation. Cultures were grown overnight in LB supplement with appropriate antibiotic and 0.2% w/v glucose to repress expression from the araBAD promoter. Protein expression from plasmid was induced in day cultures during mid-exponential phase using 0.2 % w/v arabinose unless otherwise stated.

Table 3: Media composition

Media	Contents per litre	Conditions
Gutnick minimal media	KH ₂ PO ₄ (4.7 g), K ₂ HPO ₄ (13.5 g), K ₂ SO ₄ (1.0 g), MgSO ₄ ·7H ₂ O (0.1 g)	Experimental growth media
HolE trace elements	2.85 mg H ₃ BO ₃ (Sigma), 1.8 mg MnCl ₂ ·4H ₂ O (VWR), 1.36 mg FeSO ₄ ·7H ₂ O (VWR), 1.84 mg H ₂ SO ₄ (Sigma), 1.77 mg C ₄ H ₄ Na ₂ O ₆ ·2H ₂ O (VWR), 0.5 mg Ethylenediaminetetraacetic acid ¹²⁵ (Sigma), 46.4 µg CoCl ₂ ·6H ₂ O (VWR), 26.5 µg, CuCl ₂ ·2H ₂ O (Sigma), 20.8 µg ZnCl ₂ (Sigma), 25.2 µg Na ₂ MoO ₄ ·2H ₂ O (VWR), 20 µg Na ₂ SeO ₃ (Sigma).	Supplement for experimental growth media
Luria Broth (LB)	Yeast extract (5.0 g), peptone from casein (10.0 g), sodium chloride (10.0 g) - Merck (4378013)	Experimental growth media
SOC media	Yeast extract (5.0 g), glucose (0.36% (w/v)), NaCl (0.584 g), KCl (0.186 g), Tryptone (20.0 g), MgSO ₄ (2.4 g).	Media for cloning

2.3. Growth and viability assays

2.3.1. Growth assays

Day cultures were seeded at 0.05 OD₆₀₀ in appropriate media (2.2) and grown at 37 °C unless otherwise stated. Culture volumes varied from 50 mL in 250 mL Erlenmeyer flasks to 500 µL in 48 well plates for plate reader assays. Growth was measured by OD₆₀₀ using a spectrometer or POLARstar Omega microplate reader.

2.3.2. Cell viability assays

Cell viability was measured by counting bacterial colony forming units.

1 mL of culture was sampled and centrifuged at 13,000 rpm for 3 minutes in 1.5 ml Eppendorf tubes. Bacterial pellets were washed once in sterile filtered PBS before resuspension in 1 mL PBS. Samples were serially diluted in PBS and 20 µL spotted on to LB agar plates. Plates were allowed to dry at room temperature before incubation for 16 hours at 37 °C. After incubation, colonies were counted and number of colony forming units calculated.

2.4. T7 phage lysate generation

T7 phage lysates were prepared in gutnick media for use in gutnick experiments.

Donor strains were cultured overnight in 10 mM Gutnick media at 37 °C. Overnight cultures were used to inoculate 50 mL fresh Gutnick media at an OD₆₀₀ of 0.05. Cultures were grown at 37 °C until reaching an OD₆₀₀ of approximately 0.5. Once cells had reached the appropriate OD₆₀₀, 0.4 mL of T7 phage stock lysate was added to the culture. Further incubation was carried out at 37 °C to allow lysis to occur. The culture was then sterile filtered and 10 % chloroform added before storing at 4 °C.

2.4.1. T7 phage titre

10 mM Gutnick 1.5% agarose plates were prepared in advance. Day cultures of *E. coli* MG1655 were grown in 5 mL LB. T7 phage lysate dilutions were prepared in gutnick as follows; 10⁻⁰, 10⁻², 10⁻⁴, 10⁻⁵ 10⁻⁶ 10⁻⁷ 10⁻⁸ 10⁻⁹. 30 µl of phage lysate was added to 300 µl of day culture and incubated at 37°C for 10 minutes (no shaking). After incubation, 3 mL of soft agar was added to each culture and mixed before pouring on top of pre-prepared 10 mM Gutnick plates. Plates were incubated overnight at 37 °C. The next day, phage titre was determined by counting the number of plaques formed. Each plaque is formed from one phage. The following calculation was applied to calculate plaques per mL.

Plaques/ml = # plaques x (1/ml phage plated) x dilution factor

2.4.2. P1 phage transduction

For lysate preparation, donor strains were grown overnight from single colonies in 5 mL LB. The next day, 0.2 mL donor strain was incubated 25°C with 0.4 mL P1V1R phage for 25 minutes (P1V1R neat lysate was diluted 1:10 in LB, 1% glucose, 5mM CaCl₂ solution). Following incubation, 0.25 ml of 40% (w/v) glucose, 9.25 ml of Luria broth, and 0.5 ml of 0.1M CaCl₂ were added. The cultures were left shaking at 180 rpm for 5-6 hours, until clear lysate formed with debris settled at bottom of culture. The culture was further incubated with 1 mL

chloroform for 30 minutes at room temperature. The culture was centrifuged 4000 rpm for 30 minutes. Lysate was removed and stored at 4° C.

For infection of recipient strains, donor strains were prepared as above. Recipient strains were incubated overnight in LB. 0.2 mL of recipient strain was then incubated with 0.4 mL of donor phage lysate (1:10 dilution of stock) at 37 °C for 20 minutes. Transduction was stopped by adding 0.4 ml of 1 M NaCl after 20 minutes. Cells were washed twice with 1 mL 10 mM NaCl LB before resuspending in 300 µL 10 mM NaCl LB. Reaction was plated on 10 mM NaCl LB agar plates plus relevant antibiotic and incubated overnight at 37 °C. Transduction was confirmed by colony PCR (see separate protocol).

2.5. Molecular cloning

2.5.1. DNA isolation

2.5.1.1. Genomic DNA isolation

Genomic DNA was isolated from bacteria using the Wizard® Genomic DNA Purification Kit (Promega A1120) using the manufacturers standard protocol. All centrifugation steps were carried out at 13,000 rpm for 1 minute DNA was eluted into MilliQ de-ionised H₂O before storing at -20 °C.

2.5.1.2. Plasmid DNA isolation

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen 27104). The standard manufacturers spin protocol was followed. All spin steps were carried out at 13,000 rpm for 1 minute and final elution into 30 µL MilliQ de-ionised H₂O.

2.5.1.3. PCR purification

PCR purification was carried out using MiniElute PCR purification kit (QIAGEN 28006) following the standard protocol provided by the manufacturer. PCR products were eluted in to MilliQ de-ionised H₂O and stored at -20 °C.

2.5.2. PCR

2.5.2.1. Colony PCR

Colonies were picked in to 25 µl of de-ionised H₂O before lysing at 95 °C for 5 minutes. Cells were subsequently spun at 3,000 rpm to pellet debris. For plasmids, one vector specific and one non-specific primer were used to amplify the area of interest. For genomic DNA, forward and reverse primers were designed to capture 500 base pairs up and downstream of the gene of interest.

PCR reactions were set up as per (Table 4) and run using GoTaq G2 (Promega M7848) polymerase as per programme described in Table 5. To visualise PCR results, 10 µl of PCR product was run on a 1.5% electrophoresis gel as described in 2.5.7.

Table 4: Colony PCR reagents

Reagent	1x reaction
5X green GoTaq buffer	5 µl
template	5 µl
10mM (2.5mM of each) dNTP	0.5 µl
MilliQ-water	13.39 µl
10µM forward primer	0.5 µl
10µM reverse primer	0.5 µl
GoTaq enzyme	0.125 µl

Table 5: Colony PCR reaction setup

Segment	Cycles	Temperature	Time
1	1	94°C	2 minutes
2	30	94°C	30 seconds
		55°C (or gradient)	1 minute
		72°C	1 min/kb
3	1	72°C	5 minutes

2.5.3. DpnI digestion

45µl of PCR products were incubated with 1 µL DpnI (New England Biolabs R0176L), 5 µL CutSmart buffer (New England biolabs B6004S) and 4 µL H₂O for 1 hour at 37°C. Digested DNA was removed via PCR purification (2.5.1.3).

2.5.4. Gibson Assembly

Primers for Gibson assembly cloning were designed using NEB Builder Gibson Assembly tool (www.nebuilder.neb.com). PCR to amplify backbone and insert were carried out using Q5 polymerase (See 2.5.5). Reactions were initially performed on a temperature gradient between 55 °C and 70 °C to determine the most efficient annealing temperature.

To confirm amplification of the correct size DNA, PCR products were run on 1.5% agarose TBE gels, 100 V for 30 mins and visualised using a Biorad Chemidoc. Selected PCR products were then PCR purified (see 2.5.1.3). Gibson assembly was carried out as per manufacturer

protocol (2.5.4), using 20-200 ng DNA generally at a 1:3 vector to insert ratio. If Gibson reaction failed, this step was repeated at a 1:6 or 1:10 vector to insert ratio. Gibson reaction incubations were carried out at 50 °C for 60 minutes. The product was then transformed in to DH5α cells (section 2.6.2) and incubated overnight at 37 °C on LB agar plates containing relevant antibiotic. The next day, colony PCR was carried out on individual colonies using GoTaq G2 (Promega M7848) to confirm the presence of insert (2.5.2.1). Positive colonies were grown overnight in LB plus relevant antibiotic. Plasmids were then purified (2.5.1.3) before sequencing by Genewiz.

2.5.5. PCR using Q5 polymerase

Primers were generally designed using New England Biolabs primer design software. Plasmid and genomic DNA were isolated as per separate protocols (see 2.5.1). PCR reactions were set up as per Table 6, using Q5® High-Fidelity DNA Polymerase (New England Biolabs M0491). Reactions used 10 ng of template DNA and protocol run as per Table 7. Where PCR products were to be utilised in Gibson assembly cloning, PCR products were digested using DpnI (2.5.3).

Table 6: Q5 PCR reagents

Reagent	25 µl reaction	Final concentration
5X Q5 reaction buffer	5 µl	1X
10 mM dNTPs	0.5 µl	200 µM
10 µM forward primer	1.25 µl	0.5 µM
10 µM reverse primer	1.25 µl	0.5 µM
Q5 High-fidelity DNA polymerase	0.25 µl	0.02 U/µl
Nuclease free water	Up to 25 µl	
Template DNA	Variable	10 ng

Table 7: Q5 PCR reaction setup

Segment	Cycles	Temperature	Time
Initial denaturation	1	98 °C	30 seconds
Extension time	35	98 °C	10 seconds
		55 – 70 °C	20 seconds
		72 °C	30 seconds/kb
Final extension	1	72 °C	2 minutes
Hold		4 °C	

2.5.6. Site directed mutagenesis

Primers for site directed mutagenesis were designed to be compatible with Aligent PfuUltra II Fusion polymerase (Aligent Technologies 600674) with a t_m of 55 °C. PCR reagents were set up as per Table 8 and PCR reaction carried out as per Table 9. All reactions were carried out with a t_m of 55 °C. Extension time varied depending on length of DNA.

After completion of PCR reaction, samples were treated with DpnI for one hour at 37 °C to remove template DNA. Samples were then PCR purified using MiniElute PCR purification kit (QIAGEN 28006). 5 µL of purified DNA was transformed in to DH5α cells through heat shock transformation and plated on LB agar containing appropriate antibiotic. Colonies were selected and DNA purified either through plasmid or genomic DNA extraction methods and sent for Sanger sequencing, further details of this process described in section 2.5.1.

Table 8: Site directed mutagenesis reagents

Reagent	Volume per reaction
dH ₂ O	40.5 µl
10x PfuUltra II reaction buffer	5 µl
dNTP mix (25 mM each dNTP)	0.5 µl
Forward primer (10 mM)	1 µl
Reverse primer (10 mM)	1 µl
PfuUltra II fusion HS DNA polymerase	1 µl
DNA template (100 ng/µl)	1 µl
Total reaction volume	50 µl

Table 9: Site directed mutagenesis reagents

Segment	Number of cycles	Temperature	Duration
1	1	95 °C	2 minutes
2	15	95 °C	20 seconds
		55 °C	20 seconds
		72 °C	15 seconds per kb
3	1	72 °C	3 minutes

2.5.7. Gel electrophoresis

Gels were prepared using 50 - 75 mL 1X Tris-borate-EDTA (Sigma T4415-4L), 1.5 % agarose¹²⁴ and 2.5% SYBR Safe dye¹²⁴. Samples were loaded using GelPilot DNA loading dye

(Quiagen 239901) alongside O'GeneRuler 1 KB DNA ladder (Thermofisher 11551615). Gels were run at 100 volts for 30 minutes. Gels were visualised using a BioRad ChemiDoc.

2.5.8. DNA sequencing

Plasmid and genomic DNA were isolated as per previous protocol (2.5.1). 20-100µg/mL of DNA was mixed with approximately 25pmol/µl of relevant forward, reverse, or internal primer depending on DNA length. Samples were sent to GENEWIZ UK Ltd, UK for Sanger sequencing and analysed using bioedit software.

2.6. Bacterial transformation

2.6.1. Preparation of chemically competent cells

Bacterial cells were streaked from glycerol stocks stored at -80 °C on to LB agar plates and incubated overnight at 37 °C. Single colonies were picked in to 5ml LB and incubated overnight at 37 °C. Overnight cultures were diluted 1:10 in 10 mL fresh LB and grown to an OD₆₀₀ of approximately 0.5. Cells were spun for 10 minutes, 4 °C at 4000 rpm before resuspending in competency buffer (150 mM calcium chloride, 15 % glycerol in H₂O). The washing step was repeated before resuspending remaining bacteria in 500 µL of competency media. Cells were transferred in to 50 µL aliquots and stored at -80°C.

2.6.2. Heat shock transformation

Heat shock transformations were carried out using 1 µl of relevant plasmid stock. Final concentration of between 1 and 10 µg/mL DNA was transformed into New England Biolabs (NEB) 5-alpha competent high efficiency *E. coli* cells (DH5α), XL1 blue (for cloning) or other relevant strain for *in vivo* assays. Cells were incubated on ice for five minutes and subsequently heated at 42 °C for 45 seconds before further incubation on ice two minutes.

Further incubation was carried out in 250 µl of LB broth for one hour at 37 °C before overnight incubation in 5mL LB broth.

2.6.3. Preparation of electrocompetent cells

Strains were streaked from -80 °C stocks on to LB agar containing the relevant antibiotic and incubated overnight at 37 °C. Single colonies were grown overnight at 37 °C with relevant antibiotic. Overnight cultures were diluted 1 in 100 in 50 mL fresh LB and grown to an OD₆₀₀ of approximately 0.6. Cultures were chilled on ice for 30 minutes before centrifuging at 4000 rpm for 10 minutes. Cells were washed in 25 mL 15% glycerol. Wash was repeated with 12.5 mL 15% glycerol before resuspending pellet in 80 µL 15% glycerol. Electrocompetent cells were freshly prepared on day of use, all steps carried out on ice.

2.6.4. Electroporation of electrocompetent cells

1-4 µg of DNA was incubated with competent cells and transferred to a VWR electroporation cuvette. Cells were pulsed once using a Biorad Micropulser and transferred in to 1 mL SOC media and incubated for 1.5 hours shaking 180rpm at 37 °C. After incubation, sample was spun at 7000xg for 2 minutes before removal of 950 µL of SOC media. Bacterial pellet was resuspended in remaining media and plated on LB agar containing appropriate antibiotic and incubated overnight at 37 °C.

2.6.5. Curing of antibiotic cassettes

Chemically competent cells were made as previously described. Cells were transformed via heat shock transformation with the pCP20 plasmid, recovery carried out at 30 °C for 2 hours. 50 µL of transformants were plated on LB agar containing 50 µg/mL ampicillin and incubated overnight at 30 °C. The next day, single colonies were grown in 1 mL LB at 37 °C for 3 hours followed by 44 °C for 2 hours. Cultures were diluted 1/10 in LB and plated on LB agar for overnight incubation at 37 °C.

Colonies were picked in to 500 µL LB and streaked on replica plates containing ampicillin, kanamycin and no antibiotic. Colony PCR was carried out on relevant colonies to confirm removal of antibiotic cassette.

2.7. Mass spectrometry

2.7.1. Preparation of samples

Bacteria were grown in the relevant media as per previous description. 10 mL of culture was sampled at relevant time points and washed three times in 10 mL PBS (all spins were carried out at 3000xg, 4°C for 5 minutes). Bacterial pellets were resuspended in 1 mL lysis buffer (40:40:40 acetonitrile:methanol:water) and transferred into tubes containing 0.2ml of 0.1mm acid washed zirconia beads.

2.7.2. Metabolite extraction experiments

All metabolite extraction, detection and analysis were carried out by Dr Gerald Larrouy-Maumus. The bacteria were metabolically quenched by plunging the filters into the extraction solution composed of acetonitrile/methanol/H₂O (2:2:1) precooled to 4 °C. Small molecules were extracted by the mechanical lysis of the entire bacteria-containing solution with 0.1-mm acid-washed zirconia beads for 1 minute using a FastPrep system (MP Bio®) set to 6.0 m/second. The lysates were filtered twice through 0.22-µm Spin-X column filters (CoStar®). The bacterial biomass of the individual samples was determined by measuring the residual protein content of the metabolite extracts using the BCA assay kit ¹²⁶. A 100-µl aliquot of the metabolite solution was then mixed with 100 µL of acetonitrile with 0.2% acetic acid at -20 °C,

and the mixture was centrifuged for 10 minutes at 17,000 x g and 4 °C. The final concentration of 70% acetonitrile was compatible with the starting conditions of Diamond Hydride chromatography. The supernatant was then transferred into LC/MS V-shaped vials (Agilent 5188-2788), and a 4 µL aliquot was injected into the LC/MS instrument.

2.7.3. Liquid chromatography-mass spectrometry for untargeted metabolomic analysis

Aqueous normal-phase liquid chromatography was performed using an Agilent 1290 Infinity II LC system equipped with a binary pump, temperature-controlled autosampler (set at 4 °C) and temperature-controlled column compartment (set at 25 °C) containing a Cogent Diamond Hydride Type C silica column (150 mm × 2.1 mm; dead volume of 315 µl). A flow rate of 0.4 mL/min was used. The elution of polar metabolites was performed using solvent A, which consisted of deionized water (resistivity ~18 MW cm) and 0.2% acetic acid, and solvent B, which consisted of 0.2% acetic acid in acetonitrile. The following gradient was applied at a flow rate of 0.4 ml/min: 0 minutes, 85% B; 0-2 minutes, 85% B; 3-5 minutes, 80% B; 6-7 minutes, 75% B; 8-9 minutes, 70% B; 10-11 minutes, 50% B; 11.1-14 minutes, 20% B; 14.1-25 minutes, 20% B; and 5-minutes of re-equilibration at 85% B. Accurate mass spectrometry was performed using an Agilent Accurate Mass 6545 QTOF apparatus. Dynamic mass axis calibration was achieved by continuous infusion after the chromatography of a reference mass solution using an isocratic pump connected to an ESI ionization source operated in positive-ion mode. The nozzle and fragmentor voltages were set to 2,000 V and 100 V, respectively. The nebulizer pressure was set to 50 psig, and the nitrogen drying gas flow rate was set to 5 l/minute. The drying gas temperature was maintained at 300 °C. The MS acquisition rate was 1.5 spectra/sec, and m/z data ranging from 50 to 1,200 were stored. This instrument enabled accurate mass spectral measurements with an error of less than 5 parts per million (ppm), a mass resolution ranging from 10,000 to 45,000 over the m/z range of 121-955 atomic mass units, and a 100,000-fold dynamic range with picomolar sensitivity. The data were collected in centroid 4-GHz (extended dynamic range) mode. The detected m/z data were deemed to represent metabolites, which were identified based on unique accurate mass-retention times and MS/MS fragmentation identifiers for masses exhibiting the expected distribution of accompanying isotopomers. The typical variation in the abundance of most of the metabolites remained between 5 and 10% under these experimental conditions.

2.7.4. Data analysis

Ion features were extracted using the software Agilent Profinder version B.8.0.00 service pack 3 and multivariate analyses using Agilent Mass Profiler Professional 15.1.

2.8. Antibiotic treatments

Unless otherwise stated antibiotics were supplemented to overnight cultures only at the following concentrations and were not added to media during experimental growth; chloramphenicol 12.5 ng/μl, ampicillin (AMP) 100 ng/μl and kanamycin ² 50 ng/μl.

2.9. Protein production

2.9.1. Cell free *in vitro* transcription and translation protein production

In vitro protein production was carried out by transcription translation assay using PURExpress kit (NEBE6800S), following the manufacturers protocol. For protein production, 1 μL of T7 promotor containing plasmid DNA (concentration 100 μg/mL) was added to each reaction.

For bacterial challenge experiments, single colonies were picked and grown overnight in 5 mL LB plus appropriate antibiotic. The next day, overnights were diluted 10X in H₂O and 1 μL added to each reaction. 1 μL of T7 promotor containing plasmid DNA (concentration 100 μg/mL) was added to each reaction to provide the template of the protein of interest. Samples were incubated at 37 °C for 2 hours without shaking. After incubation 2 μL of each reaction was spotted on to LB agar plates and incubated overnight at 37 °C. A further 2 μL of each reaction was added to 498 μL of LB and grown in a 48 well plate at 37 °C and growth measured by plate reader.

2.9.2. Purification of protein using AKTA pure

2 μL of plasmid DNA was transformed in to 50 μL of chemically competent JE1 (DE3) bacteria via heat shock transformation. DE3 cells are lysogenic for λ-DE3 phage and encodes the T7 RNAP under the control of the *LacUV5* promotor. JE1 cells contain a mutated *E. coli* RNAP, which is less able to bind to inhibitory proteins Gp2 and Gp5.7. Cells were plated on LB agar plus appropriate antibiotic and incubated overnight at 37°C. The next day, single colonies were used to inoculate overnight LB cultures containing appropriate antibiotic.

1 L LB cultures were prepared by diluting overnights to an OD₆₀₀ of 0.05 in LB plus antibiotic. The culture was grown to an OD₆₀₀ of approximately 0.45 before dropping the temperature to 25C for 30 mins. Protein production was then induced with 1mM IPTG (1 mL of a 1M stock solution). The cells were left to grow at 25°C for 3.5 hours before harvesting at 7000xg for 20 minutes, 4°C. The cell pellets were stored at -20 °C overnight.

The next day, cell pellets were thawed on ice and resuspended in 10 mL lysis buffer per pellet. Samples were sonicated with 10 second pulses (10 seconds on, 10 seconds off) for 10 minutes and before pelleting at 31,500xg for 45 minutes at 4 °C. Soluble sample only was

loaded on to the column. Insoluble sample was resuspended in 10 mL H₂O and frozen at -20 °C.

A 1ml HisTrap column (Cytiva) was prepared by manually washing with 3 CV water, 3 CV 0.1M EDTA, 3 CV water, 3 CV 0.1M NiCl₂, 3 CV water then 3CV buffer A.

Soluble lysate was loaded on to the column using a capillary loop, running at 1 mL/min, collecting the flow through. The column was then washed at 2 mL/min with 10 mL buffer A, 10 mL 3% buffer B (diluted in H₂O) and 10 mL buffer A. Collection was carried out in 5ml fractions. The target protein was then eluted over a 20 mL gradient from 0 to 100% buffer B, running at 0.5 mL/min, collecting in 0.5 mL fractions.

Fractions were prepared for SDS gel by mixing 15 µL fraction with 15 µL 2X LDS. Insoluble protein and samples taken before and after induction were suspended in 10 µL 2X LDS dye per 0.1 OD₆₀₀. 10 µL of prepared samples were run on a 15% SDS-page gel alongside 5 µL PageRuler pre-stained ladder (see section 2.10 for further details). Gels were run at 200V for 45 minutes.

Purified protein concentration was determined by Bradford assay following manufacturers protocol (Biorad 5000205).

Table 10: Protein purification buffers

Buffer	Final concentration
Nickel buffer A	25mM NaH ₂ PO ₄ pH 7.0, 0.5M NaCl, 5% v/v glycerol
Nickel buffer B	25mM NaH ₂ PO ₄ pH 7.0, 0.5M NaCl, 5% v/v glycerol, 1M Imidazole
Lysis buffer	Nickel buffer A + 1 EDTA free protease inhibitor tablet per 100ml (Thermofisher A32965).

2.9.3. *In vitro* transcription initiation assay

An *in vitro* transcription initiation assay was used to determine the ability of purified Gp2, R56E, Gp5.7 and R24A to inhibit RNAP transcription.

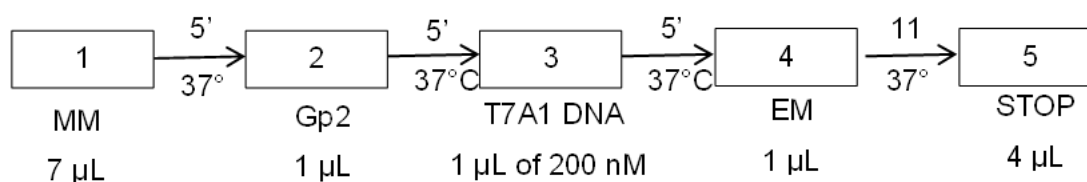
Reagents were prepared as per Table 11.

Table 11: Abortive protein activity assay reagents

Reagent	Final concentration
RB	10mM Tris pH 7.9, 40mM KCl and 10mM MgCl ₂
Master mix (MM)	100 nM E (NEB), 400 nM σ^{70} , 20U RNasin, 1X RB
Elongation mix ⁷⁴	0.5 mM CpA, 0.05 mM UTP, 32P-UTP
Stop buffer ¹²⁷	80% w/v formamide, 0.01 % w/v xylene cyanol, 0.01 % w/v bromophenol blue

The assay determines the activity of RNA polymerase, by quantifying the amount of radiolabelled transcript produced from the T7 early gene A1 promotor sequence (T7A1 DNA). The DNA probe used in this transcription initiation assay were 65 bp long, 20 of these base pairs being downstream of the +1 transcription start site.

The assay was run as per below schematic. Gp2 was pre-incubated with E σ^{70} before addition of the T7 A1 promotor. After incubation, the elongation mix was added and transcription allowed to run for 11 minutes before halting the reaction by the addition of equal volumes of *in vitro* loading dye and stop buffer (Table 11).



= minutes.

Once the reaction was complete, samples were boiled for 3 minutes and spun at 13,000 rpm for 1 minute. 20 % Urea gels were prepared by combining 10 mL UreaGel system concentrate (Geneflow A2-0002), 1.25 mL UreaGel diluent (Geneflow A2-0006), 1.25 mL 10X TBE (Lonza BE08-238210), 75μl 10% ammonium peroxydisulphate (VWR 23100-260) and 10 μl TEMED (Sigma T7024). Gels were pre-run for 30 minutes before 7 μL of sample was loaded. Gels were run at 200V for 30 minutes before transferring. Visualisation was carried out using a Typhoon.

2.10. Protein detection

2.10.1. Immunoblotting

Immunoblotting was carried out using SDS-page using Mini-protean TGX precast gels (Biorad 456-1035). Samples were loaded using 2 X Laemmli sample buffer (Sigma S3401-1VL) as loading dye and run alongside a PageRuler Plus protein ladder (Thermo scientific 26619). Gels were run at 60 volts for 15 minutes through the stacking gel followed by 100 volts for 60-90 minutes. SDS-page gels were equilibrated in transblot buffer (25 mM Tris, 192 mM glycine and 10% methanol) before transferring to 0.2 PVDF membrane using Transblot Turbo cassette (Biorad). PVDF membranes were activated in 10% methanol before transfer. Transfer was carried out at 25 volts for 30 minutes. Membranes were blocked with 5% milk in TBST for 1 hour. Primary antibody incubations were carried out overnight at 4 °C. Secondary antibody incubations were carried out for 1 hour at room temperature. Blots were developed using Amersham™ ECL™ Prime (VWR, RPN2232).

2.10.2. Antibodies

Antibody	Dilution	Secondary antibody
Primary		
Anti-mCherry (Abcam ab167453)	1:1,000	Rabbit
Anti-Gp2 (Eurogentec)	1: 1,000	Rabbit
Anti-rpoA (Biolegend, WP003)	1: 1,000	Mouse
Secondary		
Goat anti-rabbit IgG HRP (GE Healthcare NA934-1ML)	1:10,000	N/A
HRP Goat anti-mouse IgG (Biolegend, 405306)	1: 10,000	N/A

2.10.3. Coomassie blue staining

Proteins were detected by staining with Bio-Safe Coomassie Stain (Biorad 161-0787). SDS page gels were washed 3 times for 10 minutes in deionised H₂O before adding stain. Staining was carried out as per manufacturers protocol and de-stained at 4 °C overnight.

2.11. PALM microscopy

2.11.1. Sample preparation

1 mL off bacterial culture from day cultures were spun at 13,000 rpm to pellet bacteria. Cells were washed twice in 1 mL filtered gutnick media. Gutnick media was supplemented with nitrogen and carbon concentrations that reflected the concentration contained in the media at

the time point sampled. Pellets were resuspended in 10 μL of media per 0.1 OD_{600} . 1 μL of resuspended cells were placed on agarose pad and imaged immediately.

2.11.2. Agarose pad preparation

1 % agarose pads were prepared by mixing equal parts 2 % Ultrapure agarose (Thermofisher, 16500500) with 2X gutnick media. Gutnick media was prepared to reflect relevant concentrations of nitrogen and carbon for each timepoint. Agarose pads were left to dry for 30 minutes before use. Upon sample preparation, upper slides were removed from agarose pads and pads allowed to dry at room temperature before loading with bacterial sample.

2.11.3. Imaging procedure

All imaging was carried out using a PALM-optimised Nanoimager (Oxford Nanoimaging, www.oxfordni.com). pAmCherry was activated using a 561 nm laser at approximately 10 mW. In addition to 561 nm laser activation, pulses of a 405 nm laser were used to provide additional activation. For all imaging, 10,000 frames were captured at a rate of 66 frames per second. TIRF value was set to 53.5 and exposure set at 15 mS.

2.11.4. Single molecule tracking

The PALM-optimised Nanoimager software was used to track individual molecules of pAmCherry tagged proteins. The single molecule tracking function identified single molecules and their trajectories using a step distance of molecules between frames of 0.6 μM , and exclusion radius for nearest neighbour molecules of 0.9 μM . The software then calculated the apparent diffusion coefficient (D^*) of each molecule trajectory over at least four steps. D^* were sorted in to 0.01 $\mu\text{m}^2/\text{s}$ width histogram bins for further analysis.

2.11.5. Calculation of %H_{IM}

The amount of immobile Hfq molecules was quantified by calculating the %H_{IM} (Hfq immobile molecules) within the sample. It has previously been determined that the apparent diffusion coefficient (D^*) of approximately 75% of the molecules within the Hfq foci was $<0.08 \mu\text{m}^2/\text{s}$ ⁸⁹. D^* values were calculated from multiple fields of view and the proportion of these which fell into the defined immobile population was calculated. Cells containing Hfq foci contained a higher number of immobile Hfq molecules ($D^* <0.08 \mu\text{m}^2/\text{s}$), therefore have a higher %H_{IM} value.

2.11.6. Hexanediol treatment

50% hexanediol solution was freshly prepared in MilliQ H₂O and added to bacterial cultures, final concentration 10% w/v. Samples were prepared for imaging as above (2.11.1) and imaged on agarose slides containing 5% hexanediol (2.11.3).

3. **Chapter 3: Glutathione contributes to the adaptive response to nitrogen starvation in *E. coli*.**

3.1. Introduction

In chapter (1.3.2.3.2) I discussed the formation of Hfq into foci during nitrogen (N) starvation, which occurs independently of *de novo* gene expression⁸⁹. Here, we used a well established N starvation model to investigate the role of the metabolism on Hfq foci formation.

3.1.1. Bacteria become growth attenuated upon N runout: an experimental set up to study N starvation in *E. coli*

Switzer *et al.* previously established a N limiting growth model which allowed us to investigate *E. coli*'s response specifically to N starvation⁸. Batch cultures were grown in defined gutnick minimal media containing 3mM ammonium chloride as the sole N source and an excess of glucose as the sole carbon source. Figure 3.1 (left and middle panels) shows that as ammonium chloride levels were depleted, bacteria entered growth arrest in response to the complete depletion of N, whilst carbon remained present in excess⁸. After N was depleted, bacteria were still able to grow, albeit at a much slower rate, with a doubling time of 38.7 hours between N- (20 minutes after N runout) and N-24 (24 hours after nitrogen runout) compared to 1.5 hours at N+ (nitrogen replete). This continued growth demonstrated that metabolism was still active even after the external N source was depleted⁸. This model allowed us to sample bacteria at various timepoints where at defined N concentrations⁸. In this work, the main timepoints we will refer to are N+, N- and N-24. In addition to these timepoints, we also sampled at X hours after N runout, which we will refer to as N-X.

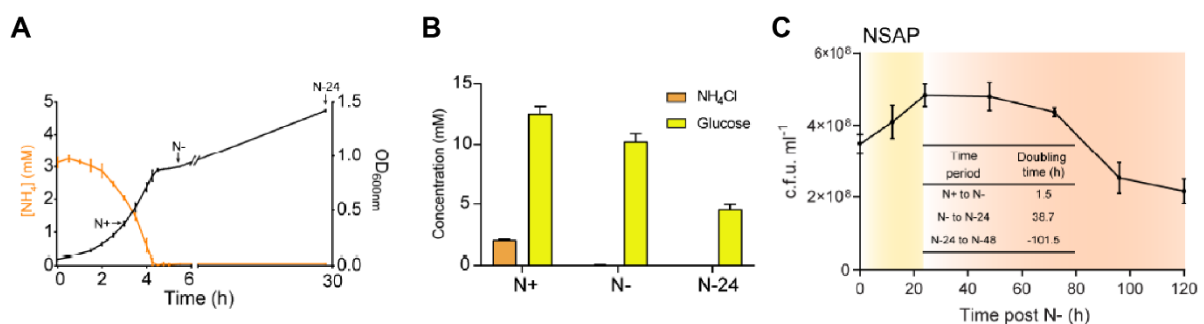


Figure 3.1: Bacteria enter growth arrest upon N runout

(A) Representative growth curve of *E. coli* measured by OD_{600nm} and concentration of ammonium chloride in growth medium as a function of time. Time points indicated are N+ (nitrogen replete), N- (20 minutes after nitrogen runout and N-24 (24 hours after nitrogen runout) (B) Concentration of ammonium chloride and glucose in growth media at N+, N- and N-24. (C) Viability of *E. coli* over 5 days during N starvation, measured by counting colony forming units. Figure adapted from Switzer *et al.* 2020⁸

This published model was used to discover and characterise the Hfq foci discussed in the introduction (1.3.2.3.2) ⁸⁹. This model was therefore the most appropriate to use to continue to investigate the Hfq foci.

3.1.2. Chapter Aim

Broadly in this work we aimed to elucidate the molecular trigger for the formation of Hfq foci in *E. coli*. As discussed in chapter (1.3.2.3.2), it has been established that Hfq foci form independently of transcription and translation, we therefore reasoned that formation of the Hfq foci could be triggered by metabolic changes instead. Here, we aimed to explore the role of cellular metabolism on Hfq foci formation, and whether Hfq foci are linked to the survival of *E. coli* during N starvation.

3.2. Glutathione contributes to the adaptive response of *E. coli* during N starvation

3.2.1. Metabolomics revealed key components of the glutathione biosynthesis pathway were upregulated during N starvation

To determine the role of the metabolism on the formation of Hfq foci, we first wanted to identify metabolomic changes which occurred during N starvation. Samples were collected by Lynn Burchell at key timepoints during N starvation; N+ (N replete), N- (short term N starved), and additional timepoints (N-3, N-12, N-18) until long term N starvation at N-24. All LC-MS was conducted and analysed by Dr Gerald Larrouy-Maumus. Analysis cut off was set at 2-fold changes, with adjusted p-value of less than 0.05.

Unsurprisingly, the metabolomic analysis demonstrated broad changes over the course of N starvation (Figure 3.2 A). Interestingly, there was an overall increase in glutathione levels, as well as other key components of the glutathione biosynthesis pathway (Figure 3.2 A & B). We observed that glutathione was upregulated during the early stages of N starvation and remained high throughout (Figure 3.2 C). As over 30% of the metabolites necessary for components of the glutathione biosynthesis pathway were enriched (Figure 3.2 B), we chose to investigate this further.

The role of glutathione has previously been studied in stress conditions including oxidative, osmotic and low pH. Glutathione has been shown to play a role in survival during N starvation, however the mechanism has yet to be fully characterised ¹²⁸. This thesis will investigate the physiological role of the glutathione during N starvation; principally by characterising phenotypic differences of the previously published N starvation marker, the Hfq foci. We reasonably hypothesise if glutathione is being upregulated in response to N starvation this may influence Hfq foci formation. As discussed in (1.3.2.3) many of physiological aspects of

Hfq foci formation have been identified and therefore can be studied and compared in the absence of glutathione.

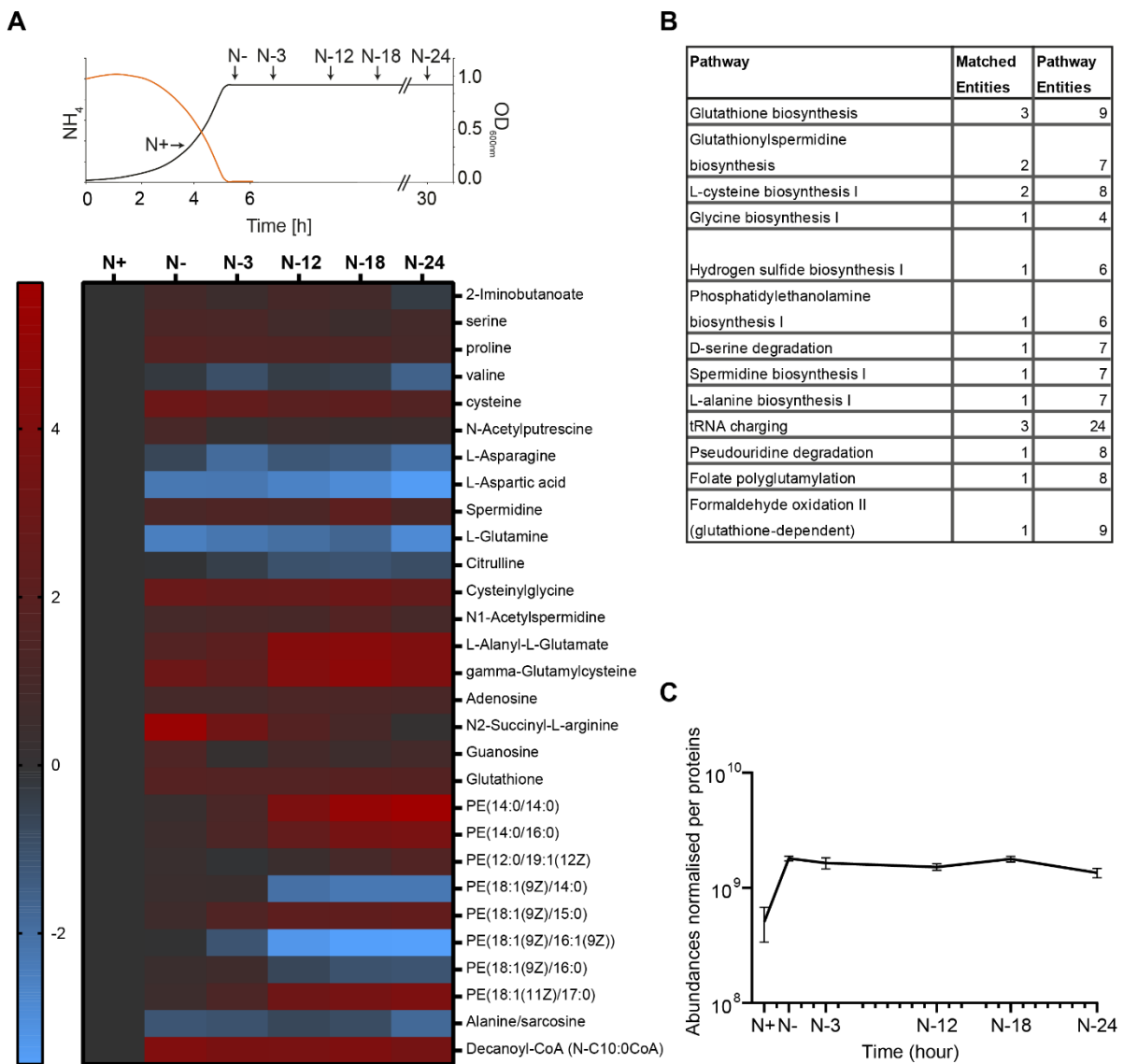


Figure 3.2: Metabolic changes during N starvation in *E. coli*

(A) Schematic showing N starvation model including timepoints sampled for mass spectrometry. Heat map showing log₂ fold changes of selected metabolites over time during N starvation (timepoints indicated) in WT *E. coli*. All changes relative to N+. Cut off log 2-fold change adjusted P value less than 0.05. (B) Table showing total number of matched entities in metabolic pathways. Table is ordered from top to bottom from highest percentage of matched to total entities to lowest, including only matches over 10%. (C) Graph showing abundance of glutathione over time during N starvation in WT *E. coli*. Error bars represent standard deviation (n=3).

3.2.2. Glutathione

Glutathione (GSH) is the most abundant low molecular weight, non-protein thiol found in proteobacteria¹²⁹. Glutathione exists in two forms, reduced glutathione (GSH) and oxidised glutathione (GSSG)¹³⁰. In prokaryotes, GSH is found mainly in gram negative bacteria including *E. coli*¹²⁸. GSH is a tripeptide containing glycine, glutamate and cysteine, with a molecular mass of 307 Da, and contains two negatively charged carboxyl groups and a positively charged amino acid group¹²⁸. GSH is an abundant component of the *E. coli* cytosol, with physiological concentrations ranging from 0.1 mM to 10 mM¹³¹⁻¹³³.

3.2.2.1. Biosynthesis and degradation of glutathione in *E. coli*

GSH is synthesised in two sequential reactions, catalysed by the enzymes γ -glutamyl-cysteine synthetase (GCS) and glutathione synthetase⁵ (Figure 3.3)⁵. GCS is a monomer of 58.3 Da encoded by the *gshA* gene¹³⁴. GS is a tetramer consisting of four identical subunits of 36 kDa encoded *gshB*¹³⁵. During the first GSH synthesis reaction, GCS catalyses the formation of a peptide bond between the γ -carboxyl group of glutamate and α -amino group of cysteine, forming γ -glutamyl-cysteine. This first reaction is the rate limiting step of glutathione production and is governed by the concentration of cysteine relative to glutamate. In the second reaction, GS catalyses a peptide bond between the α -carboxyl group of γ -glutamyl-cysteine and the α -amino group of glycine to form γ -glutamyl-cysteine-glycine (Figure 3.3).

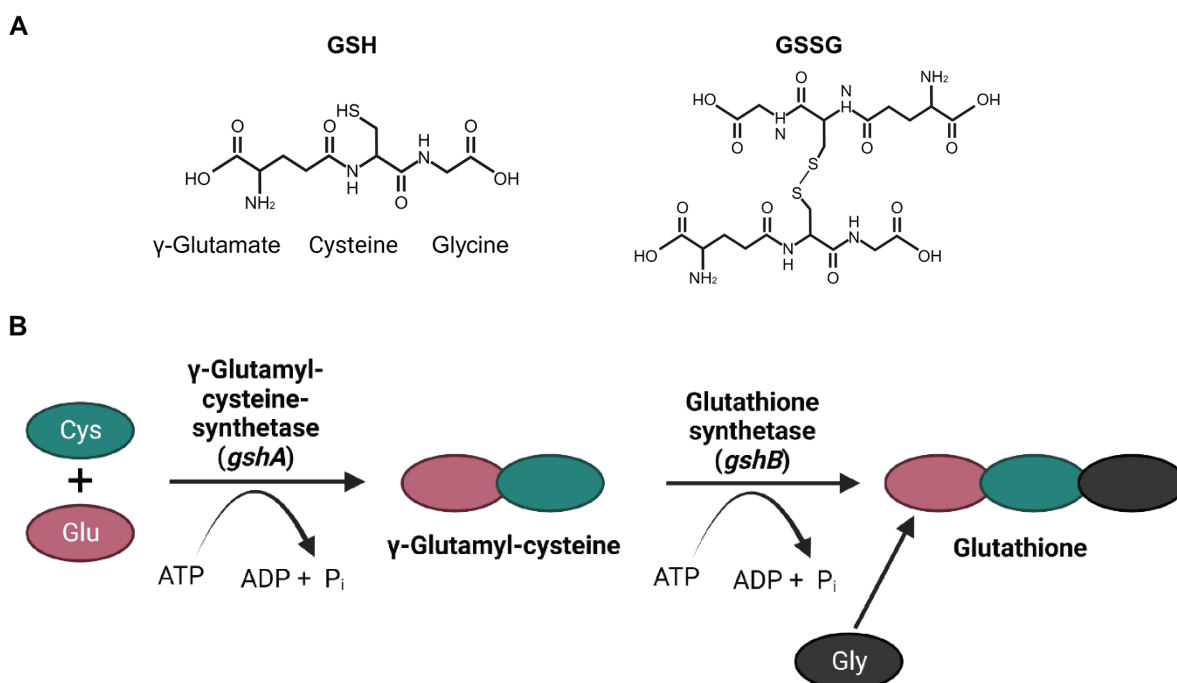


Figure 3.3: Glutathione structure and biosynthesis in *E. coli*

A) Chemical structure of GSH and GSSG.

B) Glutathione is synthesised in two steps, catalysed by γ -glutamyl-cysteine-synthetase (GCS) and glutathione synthetase⁵. See text for details.

Only one type of peptidase is known to break down glutathione, γ -glutamyl transpeptidase (GGT). GGT interacts with glutathione on the outer side of the plasma membrane where it cleaves the gamma link between cysteine and glutamate, transferring the glutamyl residue to another amino acid. The remaining product of this enzymatic reaction, L-cysteinyl-glycine, undergoes further cleavage to cysteine and glycine which are then absorbed into the cell by an unknown mechanism ^{128, 136}. Here, a large proportion of cysteine is incorporated back into glutathione. The remaining cysteine pool is incorporated into amino acids for use in protein synthesis depending on the requirements of the cell ¹³⁶.

Detail on breakdown of glutathione by GGT are further discussed in (4.1.1).

3.2.2.2. Glutathione homeostasis and status

Maintenance of GSH concentration depends on a highly dynamic balance between synthesis, degradation, oxidation, reduction and transport ^{128, 129}. The mechanisms involved in GSH homeostasis are currently not well understood. Current literature suggests that GSH concentrations are controlled in the cytoplasm by three factors. Under normal physiological conditions GCS is feedback inhibited by GSH, with GSH binding both to the glutamate binding site in the active site of GCS, as well as a separate position which binds to the thiol group of GSH ¹³⁴. This feedback inhibition maintains physiologically constant concentrations of GSH within the cell. Because of this, GSH production is largely independent from the level of *gshA* expression ¹²⁹. Therefore, feedback inhibition and cysteine concentration (previously discussed) are the two primary factors controlling GSH levels within the cell ⁵. Another potential way bacteria can control the intracellular concentration of GSH is through hydrolysis of GSH by the enzyme GGT, discussed in detail (4.1.1). However, GGT is a periplasmic protein and hydrolysis of GSH requires for efflux of GSH into the periplasm, and there is currently no evidence that GGT plays a significant role in control of cytoplasmic GSH levels. GSH is known to undergo transmembrane cycling in growing *E. coli*, however function of this phenomenon has not been confirmed ^{137 138}. There is also no evidence pertaining to the role of GSH in the periplasm or if it exists there under physiological conditions.

GSH can be transported into the cell from extracellular space by the ATP dependent yliABC transporter ¹³⁹, it has been demonstrated bacteria uptake GSH and used it as a sole source of sulphur, as well as utilising GSH as a cysteine and glycine source ¹³⁶ suggesting that GSH can be scavenged and utilised as a nutrient source. As this import appears to be to scavenge nutrients, rather than GSH as a whole molecule, it is unlikely that this mechanism plays a significant role in maintaining GSH levels in the cell unless bacteria are unable to produce GSH endogenously.

Glutathione can exist in cells in a variety of forms; the most abundant of these being reduced (GSH), oxidised (GSSG) and mixed disulfide (Figure 3.3) ¹²⁸. Reduced glutathione (GSH) is oxidized into glutathione disulfide (GSSG) ¹³⁰ - two GSH molecules linked together at their sulphur atoms by a disulfide bond. Oxidation of GSH to GSSG and GSH to form mixed disulfides are reversible reactions, with reduction catalysed by glutathione reductase (GOR) and glutaredoxins (grx). GSSG is extremely toxic, as it reacts easily with free sulfhydryl groups, it is therefore important for the cell to reduce GSSG through GOR. The importance of this reaction is reflected by the highly conserved nature of this protein, which is widespread and homologous in bacteria, fungi, protozoa, animals and plants ¹²⁸. Disulfides and mixed disulfides are reduced by glutaredoxins using GSH, producing a disulfide containing GSH, which undergoes further reduction by GOR ¹⁴⁰.

In eukaryotes, GSH status of a cell can be defined by the total concentration and ratio of reduced to oxidised glutathione (GSH:GSSG). In *E. coli*, approximately 99.5% of glutathione exists in its reduced state (GSH). Whilst the ratio of GSH:GSSG is a useful tool to quantify the redox state of a cell in eukaryotes, this does not appear to be the case for prokaryotes. Data regarding GSH:GSSG status in prokaryotes is conflicting, and therefore cannot always be used to indicate the redox state of bacteria ¹²⁸.

GSH homeostasis and status differs depending on condition and stress experienced by bacteria, which we will discuss in detail below.

3.2.2.3. Roles of glutathione in bacteria

Whilst the role of GSH is well characterised in eukaryotes, much less is known about the molecular basis of its protective role in prokaryotes. Glutathione has been observed to increase upon entry into stationary phase ¹³¹, and is required for growth in specific conditions. GSH participates in the maintenance of redox balance within the cytoplasm, defence against toxic compounds, stress responses and has recently been implicated in bacterial virulence ¹⁴¹. Much of the data pertaining to GSH's role in stress responses are conflicting and heavily dependent on growth stage and conditions. *E.coli* accumulates GSH to differing degrees in many stress conditions, however no data exist determining how GSH levels are monitored or altered in response to these stressors. In many conditions, it is often unclear what benefit accumulation, or cycling, of GSH affords the bacteria. As discussed above, production, maintenance and degradation of GSH is extremely multifaceted and the many of the cellular processes governing GSH levels and redox status are still unclear in prokaryotes.

3.2.2.3.1. Oxidative stress

Cells experience oxidative stress when they are exposed to increased levels of reactive oxygen species ^{142, 142, 143} such as hydroxyl radicals (OH[•]), hydrogen peroxide, superoxide anions (O₂^{•-}) and hydroperoxides (ROOH) ^{128, 144}. ROS are highly reactive and cause damage to all macromolecules, making them a significant threat to cells. ROS induced damage includes double stranded DNA and RNA breaks, peroxidation of lipids and carbonylation of proteins. Proteins such as pyruvate kinase, DnaK, glutamate synthase, glutamine synthetase and some TCA cycle enzymes have been shown to be particularly susceptible to carbonylation (oxidation of amino acid residues) initiated by ROS during stationary phase ¹⁴⁵.

Bacteria have evolved complex response and repair mechanisms to oxidative stress ¹⁴⁴. The adaptive response to oxidative stress is facilitated by OxyR and SoxRS, transcription factors which induce expression of antioxidant substances. When bacteria experience oxidative stress, OxyR undergoes major conformational changes; activated OxyR induces the expression of genes which encode enzymes, efflux pumps and the SOS response. Interestingly, OxyR induces the expression of glutathione reductase ¹⁴⁶ and glutathione S-transferase I (*grxA*) ^{147, 148}, implicating GSH in protecting against oxidative stress. GSH has reducing properties and is able to eliminate free radicals through the sulfhydryl group of cysteine, which serves as an electron donor ¹⁴². GSH also plays an indirect role by reducing OxyR through *grxA* in the presence of GSH, leading to the inactivation of OxyR *in vitro* and *in vivo* ^{129, 149, 150}. GSH synthesis enzymes are not regulated by transcription factors and expression is not altered in response to H₂O₂ or superoxide stress but seem to be regulated indirectly in response to H₂O₂ independently of OxyR ^{151, 152}.

Susceptibility of mutants with compromised GSH production to H₂O₂ and superoxides appears to be dependent on growth stage and conditions ^{153, 128}. It has been demonstrated GSH null mutants have normal resistance to H₂O₂ challenge during exponential phase ¹⁴³ and only become susceptible to H₂O₂ during stationary phase ¹³⁰. Whilst GSH:GSSG ratio is an accurate indicator of redox state in eukaryotes ¹²⁸, addition of H₂O₂ has been shown to affect the GSH:GSSG ratio in growing *E. coli* only at lethal concentrations of over 25 mM ¹³⁰, suggesting the ratio of GSH:GSSG is a low fidelity indicator of the redox state of the cell in prokaryotes.

The susceptibility of GSH deficient strains to ROS is not only significantly dependent upon growth phase, but also growth conditions ¹⁵³. This, along with the overlapping components of the antioxidant system are why the reported roles of glutathione in oxidative stress are ambiguous and open to further investigation. Although the mechanisms are debated, it is clear GSH plays an integral role in the prokaryotic response to oxidative stress.

3.2.2.3.2. Glutathionylation

Glutathionylation is a post translational modification where mixed disulfides are formed between protein cysteines and glutathione. This phenomenon is mainly found in cells experiencing oxidative stress. In *E. coli*, approximately 2% of glutathione exists in a mixed protein-disulfide state ¹⁵⁴. Glutathionylation occurs through direct oxidation of either a protein thiol or GSH, followed by formation of a mixed disulfide. Alternatively, glutathionylation can occur through thiol-disulfide exchange between GSSG and a protein cysteine ^{129, 155}. The literature suggests glutathionylation protects proteins against over-oxidation during oxidative stress, allowing them to be efficiently reduced to back their native state by glutaredoxins once oxidative environment has returned to equilibrium ^{156 157}. Interestingly, glutathionylation is thought to have a regulatory effect on some proteins ^{156, 158}. Whilst this is a very common form of redox regulation in eukaryotes, very few proteins have been identified undergoing this type of regulation in prokaryotes ¹⁵⁹.

In *E. coli*, a small number of proteins have been identified which are regulated through glutathionylation. Reduced methionine production during oxidative stress is due to inactivation of MetE via glutathionylation ¹⁵⁶. PAPS reductase (required for growth when sulfate is the only sulphur source) is also inactivated by glutathionylation during oxidative stress - it is proposed that this shut down conserves reducing equivalents under oxidative conditions ¹⁵⁸. Whilst it is known that glutathionylation has a regulatory effect on some prokaryotic proteins *in vivo*, more research is required to determine the extent and function of this mode of regulation.

3.2.2.3.3. Osmotic stress

To adapt to osmotic stress, prokaryotes have developed systems to regulate turgor pressure within the cell ¹⁶⁰. During the initial response to hyperosmotic stress, *E. coli* maintains turgor pressure by accumulating high amounts of potassium (K⁺) ions and glutamate. This primary response eventually has detrimental consequences for the cell, as accumulation of these molecules past a certain threshold disturbs the ionic balance of the cytoplasm and inhibits enzyme activity ^{161, 162}. Bacteria have therefore developed secondary mechanisms to control turgor pressure in response to high osmolarity, this process involves accumulating osmolytes such as trehalose, glycine, proline and betaine, which do not inhibit enzymatic activity due to their neutral charge ^{160, 163, 164}.

GSH is a metabolite which becomes elevated during hyperosmotic shock in *E. coli* with a negative charge at physiological pH ¹⁶¹. Changes in the extracellular levels of GSH have also been observed in *E. coli* in elevated osmotic conditions; this is a rapid decrease in the concentration of internal GSH during initial stages, followed by subsequent elevation during later stages. *E. coli* deficient in GSH production show compromised growth in high osmotic

conditions ¹³³, and bacteria which do not contain GGT (and therefore cannot break down glutathione) have been shown to have a higher propensity for osmoadaptation ¹⁴⁶. Whilst it is clear from this evidence that GSH plays an important role in protecting against hyperosmotic stress, the underlying mechanism is currently unknown. GSH has been shown to regulate K⁺ export ¹⁶⁵, however the effects of osmoadaptation are not due to changes in K⁺ retention ¹³³. It is suggested that the role of GSH during osmotic shock may be related to its role as a protectant against oxidative stress, as osmotic shock in *E. coli* is often accompanied by responses common to oxidative stress ¹⁶⁶. Therefore, GSH may be part of an oxidative stress response induced by osmotic shock ¹⁴⁶, however the molecular basis of this is still unclear and this hypothesis is yet unproved.

3.2.2.3.4. Low pH and regulation of intracellular potassium

GSH plays a role in maintaining cellular pH and protecting against acidic conditions ¹²⁹. Efflux of potassium (K⁺) has been linked to lower cytoplasmic pH ¹⁶⁷, and pH homeostasis has been proposed to be dependent on K⁺ and Na⁺ transport ¹⁶⁸. Under low pH conditions, *E. coli* upregulates expression of the high affinity K⁺ uptake system, Kdp ^{169, 170}, and mutants unable to produce GSH have decreased cytoplasmic pH when grown in low K⁺ media ¹⁶⁷. It has also been shown that GSH inhibits K⁺ export channels KefB and KefC, and in the absence of GSH K⁺ leaks out of the cell ^{165 171}. Together this data suggests that the protectant effect of GSH during low pH may be due to its regulation of K⁺ levels within the cell.

3.2.2.3.5. Protection from methylglyoxal and chlorine compounds

Methylglyoxal (MG) is a toxic electrophile. In *E. coli* MG is produced during unbalanced sugar metabolism, when switching from low levels of sugar to high ^{172, 173}. MG production is primarily from dihydroxyacetone phosphate via an energetically unfavourable pathway, by the enzyme methylglyoxal synthase which bypasses glycolysis reactions ^{172 174}. The toxicity of MG is thought to be due to its interaction with the nucleophilic centre of DNA and its ability to covalently modify lipids and proteins ^{175, 176}. Interestingly, modification of arginine residue of glutamine synthetase (an essential protein in N metabolism) by MG results in the complete inactivation of the protein, with the modified enzyme appearing to be extremely stable ¹⁷⁶.

GSH plays a significant role in the protection against MG toxicity ¹⁶⁷. GSH is required for MG detoxification by glyoxalase I and II. Depletion of GSH by glyoxalase I and II results in activation of KefB and KefC, increasing efflux of K⁺ and reducing intracellular pH ¹⁶⁷. It is therefore suggested that GSHs role in protecting against MG involves lowering cytoplasmic pH, which provides cross protection against the toxic effects of MG ^{167, 172}. GSH deficient mutants leak K⁺, thereby reducing intracellular pH, but are more sensitive to MG than parent strains - demonstrating that acidification of the cytoplasm in the absence of GSH is not enough

to protect cells against MG ^{131 167}. The mechanism by which GSH and low pH protects *E. coli* against the effects of MG is not currently known, although it has been proposed that it may be that low pH activates DNA repair mechanisms or prevents MG from interacting with macromolecules ¹⁷².

GSH protects against chlorine compounds hypochlorous acid ^{177 177} and monochloramine (NH₂Cl) by direct interaction to produce less harmful substances ¹⁷⁸. It is suggested that this reaction occurs spontaneously ¹⁷⁸. It has also been shown that HOCl provokes a cellular response similar to that seen upon H₂O₂ challenge ^{177, 179}, presenting the possibility that GSH is only protecting against chlorine compounds indirectly via its role in oxidative stress.

3.2.3. Deletion of *gshA* and *gshB* genes in *E. coli* results in undetectable levels of glutathione

To investigate the role of glutathione during N starvation and determine whether it played a role in Hfq foci formation, we created a mutant strain lacking the genes encoding both enzymes in the glutathione biosynthesis pathway. These enzymes, *gshA* and *gshB*, encode γ-glutamyl-cysteine-synthetase and glutamine synthase respectively.

To confirm that our mutant strain ($\Delta gshAB$) could not produce glutathione, we measured glutathione levels at N-24 using targeted LCMS in both WT and $\Delta gshAB$ strains. As expected, glutathione was abundant in WT bacteria during long term N starvation and only negligible amounts were detected in the $\Delta gshAB$ mutant (Figure 3.4). Having confirmed the absence of glutathione production in the $\Delta gshAB$ mutant strain, we proceeded to characterise $\Delta gshAB$ to determine whether the absence of glutathione affected Hfq foci formation.

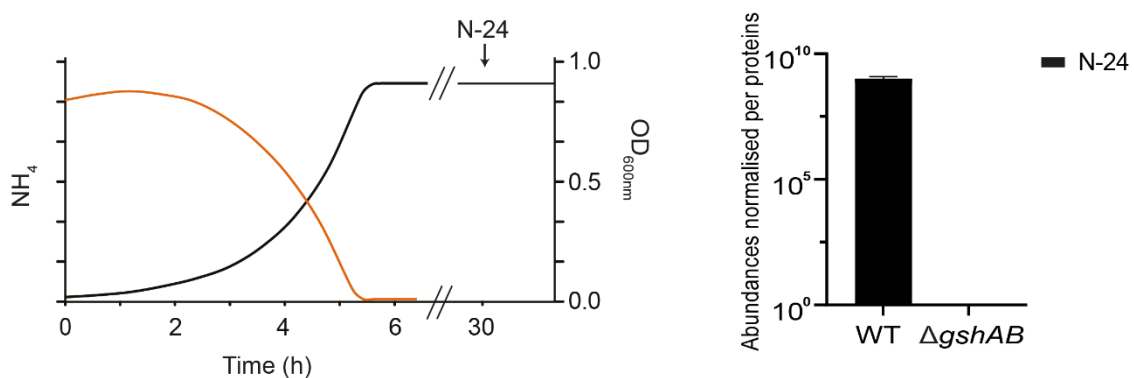


Figure 3.4: WT and $\Delta gshAB$ glutathione levels at N-24

Schematic showing N starvation model including timepoints sampled (left). Abundance of glutathione detected by targeted mass spectrometry in WT and $\Delta gshAB$ *E. coli* at N-24 (right graph) normalised per proteins. Error bars represent standard deviation (n=3).

3.2.4. Glutathione is not required for optimal growth or survival of *E. coli* during N starvation

To assess the importance of glutathione during N starvation, we initially established if glutathione had any effect on bacterial growth. When $\Delta gshAB$ were grown in our model N starvation conditions, we saw no notable differences in the initial growth dynamics between $\Delta gshAB$ and WT strains. Both strains had similar lag and doubling times, with both reaching N run out around 5 hours 45 minutes (Figure 3.5 A). It was clear glutathione was not essential for initial optimal growth in our N starvation conditions. We similarly observed no significant differences in survival between the WT and $\Delta gshAB$ mutant during the first 24 hours of N starvation (Figure 3.5 B).

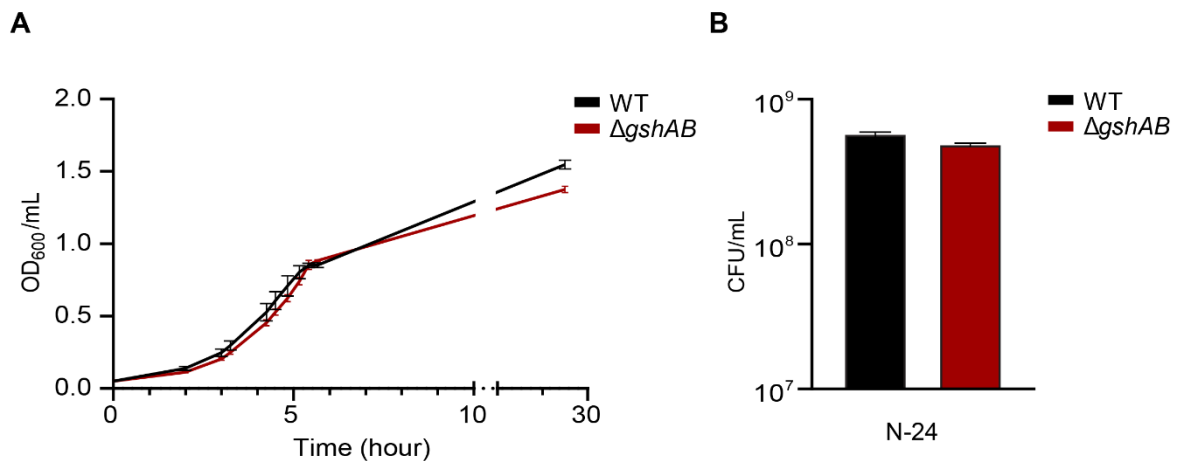


Figure 3.5: Growth and survival of WT and $\Delta gshAB$ *E. coli* (A) Growth measured by OD₆₀₀nm of WT and $\Delta gshAB$ *E. coli* during N starvation. (B) Viability of WT and $\Delta gshAB$ *E. coli* at N-24, measured by counting colony forming units. Error bars represent standard deviation (n=3).

3.2.5. Individual Hfq molecules form foci faster in the absence of glutathione

As previously discussed in chapter (1.3.2.3) individual molecules of Hfq assemble into distinct foci and are a prominent feature during N starvation. We therefore used Hfq foci as a marker of cellular stress during N starvation in *E. coli*. As we previously observed an increase in glutathione during N starvation (3.2.1), we reasoned that glutathione may have an effect on the Hfq foci. To investigate the role of glutathione in Hfq foci formation, we used live-cell PALM (photoactivated localisation microscopy) to image $\Delta gshAB$ throughout N starvation.

PALM microscopy is a fluorescence microscopy imaging technique which allows the user to obtain images of tightly packed molecules. Due to the diffraction limit of light, traditional

fluorescence techniques are limited in their resolution. This resolution can be improved through techniques such as PALM which utilises stochastic activation of photo-activated fluorescent molecules upon excitation with a specific wavelength of light. These fluorophores emit for a short period of time before photobleaching. Individual fluorescence events can then be used to calculate the localisation of the fluorophore at nanometre resolution; subsequent images can then be taken to develop high-resolution images of the localisation of thousands of individual fluorophores (i.e., tagged proteins of interest) within the same bacterial cell. When used in experiments with live cells, PALM is a tool by which we can track the diffusion of individual molecules over a time course and understand their behaviour. Here, we used PAmCherry (photoactivatable mCherry) which is non-fluorescent until excited with 350 - 400 nm light. We detected fluorescence using a Nanoimager benchtop super resolution microscope, which identifies individual molecules and calculates their diffusion around the cell (the apparent diffusion coefficient or D^*). From this data we could quantify and compare D^* of Hfq in different strains and conditions, as well as calculating the %H_{IM} (proportion of total Hfq molecules which are within the immobile population) within the sample. As molecules with the Hfq foci were relatively immobile, cells with Hfq foci contained a higher number of immobile Hfq molecules (defined previously as $D^* < 0.08 \mu\text{m}^2/\text{s}$), therefore have a higher %H_{IM} value (see methods for further information).

As expected, Hfq was distributed evenly around WT cells at N+ (Figure 3.6). Similar distribution was also observed in $\Delta gshAB$ *E. coli*. In addition to the visual similarity, the %H_{IM} values of both $\Delta gshAB$ and WT strains were approximately the same (26% and 28% respectively) at N+.

Interestingly, at N- we observed slight clustering of Hfq in $\Delta gshAB$, accompanied by a slight increase in %H_{IM} value compared with the WT (36% and 31% respectively) (Figure 3.6). Following 3 hours of nitrogen starvation (N-3), we observed distinct foci forming in the $\Delta gshAB$ mutant bacteria not seen in the WT. At N-3, the %H_{IM} had increased to approximately 45% in the $\Delta gshAB$ strain compared to 30% in the WT, with the WT strain only reaching a similar value of 51% and being visually similar by N-24. By N-24 $\Delta gshAB$ foci appeared denser and had a higher %H_{IM} value than the WT, showing that a larger proportion of Hfq was immobile in the foci within the mutant cells (Figure 3.6). This data demonstrated that in the absence of glutathione, the Hfq foci form early in our N starvation condition. This thesis will explore how and why the foci form early in the absence of glutathione.

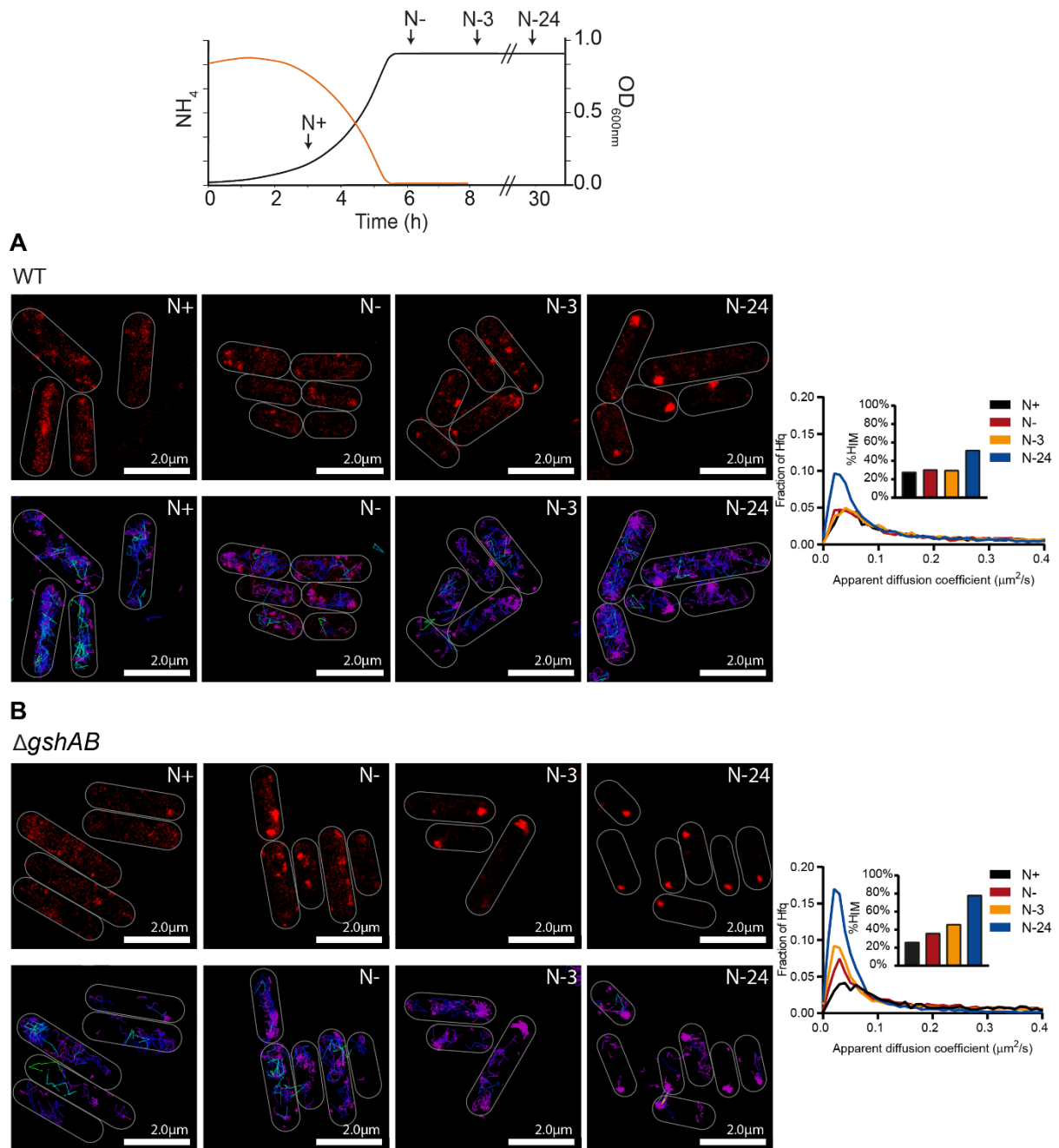


Figure 3.6: Hfq foci form sooner in $\Delta gshAB$ *E. coli* Schematic depicts N starvation model including timepoints sampled. (A-B) Representative PALM images (upper panels) and single molecule tracks (lower panels) of Hfq in *E. coli* during N starvation (A) WT, (B) $\Delta gshAB$. Images taken at indicated timepoints. Graphs display the apparent diffusion coefficient of Hfq, inset bar graphs show corresponding %HIM value.

3.2.6. The absence of glutathione does not affect Hfq levels in *E. coli* during N starvation

It is well established in the literature that LLPS of proteins can be influenced by protein concentration^{48, 52}. As the Hfq foci are a liquid-liquid phase separated structure, we wished to establish whether early formation of Hfq foci in $\Delta gshAB$ was simply due to altered liquid-liquid phase separation attributed to an increase in Hfq protein levels.

We grew both WT and $\Delta gshAB$ strains in our N starvation condition, sampling at three main timepoints (N+, N- and N-3). We did not analyse further timepoints as the phenotypic difference in Hfq foci formation could clearly be observed by N-3.

Hfq was detected via western blot using an antibody against mCherry, with rpoA used as a loading control (which our lab had previously established does not change during N starvation). We found that the protein levels of Hfq in our $\Delta gshAB$ mutant were comparable to that of WT during N starvation (Figure 3.7). We therefore concluded that the early Hfq foci formation observed in $\Delta gshAB$ *E. coli* was not due to a difference in Hfq protein concentration from WT.

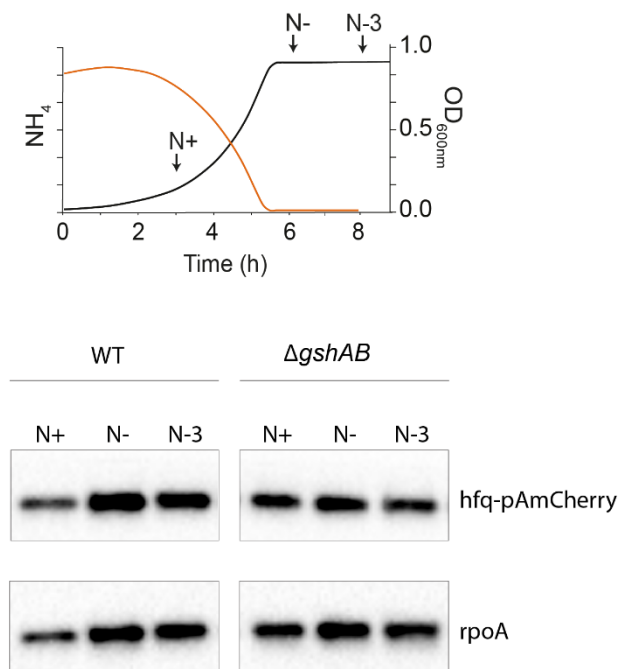


Figure 3.7: Hfq levels are comparable between WT and $\Delta gshAB$ *E. coli* throughout N starvation

Top graph depicts N starvation model including timepoints sampled. Representative immunoblot of whole-cell extracts of WT and $\Delta gshAB$ *E. coli* with hfg (MW 8 kDa) chromosomally tagged with PAmCherry: cells sampled during N starvation at N+, N- and N-3 (probed with anti-PAmCherry antibody and anti-rpoA (as loading control)).

3.2.7. The absence of glutathione does not have a universal effect on proteins known to undergo liquid-liquid phase separation

After observing the visual difference in behaviour of Hfq in our $\Delta gshAB$ strain compared to WT, we considered if this phenomenon had a universal effect on all proteins known to undergo liquid-liquid phase separation or if it was specific to Hfq. As discussed previously in this chapter, glutathione protects the bacterial cell from an array of stresses, including osmotic and oxidative stress and low pH. Many of these stresses have been shown to affect properties which influence LLPS of proteins containing intrinsically disordered domains ¹²⁶, we reasoned that the absence of glutathione may have a general effect on LLPS separable protein within the cell.

We compared the behaviour of RNA polymerase subunit β' and ribonuclease E (RNase E) in WT and $\Delta gshAB$ *E. coli* strains. These proteins have been shown to liquid-liquid phase separate during N starvation ^{84 96}, we therefore reasoned these would be good candidates to study. To visualise our proteins of interest, we used PAmCherry tagged RpoC and Rnase E WT and $\Delta gshAB$ *E. coli* strains, taking PALM images during N starvation at N+, N-, N-3 and N-24.

Consistent with the literature, we observed small clustering of RpoC at N+, during exponential growth (Figure 3.8 A). We saw no visible difference in the clustering of RpoC between WT and $\Delta gshAB$ mutant *E. coli* during the initial stages of N starvation (Figure 3.8 A). By N-24 we saw a subtle difference in the apparent diffusion coefficient of RpoC, observing an increase in the amount of faster diffusing molecules. However, this difference in diffusion coefficient was the opposite effect of the phenomenon we detected with Hfq and we did not observe any visual differences in RpoC between the two strains. We therefore did not investigate this increase in RpoC diffusion, as it was outside the scope of this work.

RNase E has been shown to form small clusters around the periphery of the cell under certain conditions ⁸⁴. When grown under our N starvation conditions, we also observed clustering of RNase E in both the WT and $\Delta gshAB$ (Figure 3.8 B). However, there was no detectable visible difference in this clustering between the two strains. This was reflected by similar patterns of movement shown by apparent diffusion coefficient at all timepoints.

From this data concluded that glutathione does not have a universal effect on LLPS separated proteins in *E. coli* during N starvation, and that the effect on the Hfq foci formation may be somewhat specific to Hfq.

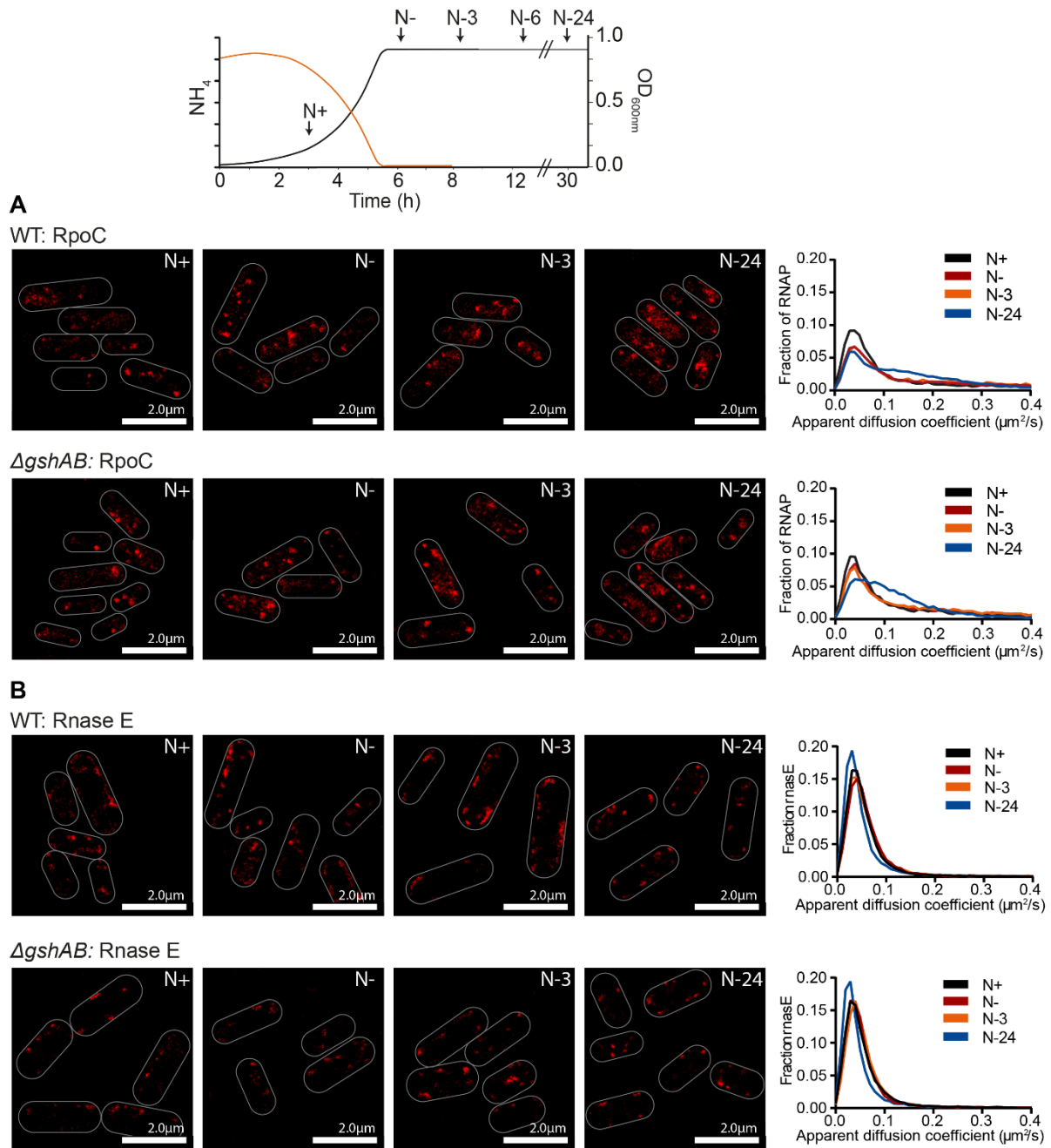


Figure 3.8: LLPS of RpoC and RNase E during N starvation in WT and $\Delta gshAB$ *E. coli*

Top schematic depicts N starvation model including timepoints sampled. (A, B) Representative PALM images of (A) RpoC and (B) RNase E in WT and $\Delta gshAB$ *E. coli* during N starvation. Images taken at indicated timepoints. Graphs display the apparent diffusion coefficient of Hfq, inset bar graphs show %H_{IM}.

3.2.8. Hfq foci do not disperse upon relief of N starvation in $\Delta gshAB$ *E. coli*

We know that the Hfq foci form by LLPS in response to N starvation, and that relief of N stress disperses the foci⁸⁹. Reversibility is a prominent feature of the Hfq foci, see (1.3.2.3.2) for further detail.

Due to the differing formation dynamics of the Hfq foci in our $\Delta gshAB$ mutant, we reasoned that the dispersal dynamics may also differ from WT. As discussed in section 1.3, we know that the characteristics of LLPS structures can be affected by different stressors, and these proteins can exist in a number of different states. Having determined Hfq foci formation dynamics in the $\Delta gshAB$ background are somewhat specific to Hfq, we considered whether Hfq foci in $\Delta gshAB$ were irreversibly changed during N starvation. To determine this, we investigated the dynamics of Hfq foci dispersal in $\Delta gshAB$.

We recovered N-24 $\Delta gshAB$ *E. coli* in fresh Gutnick media containing 10mM ammonium chloride and observed the foci over a 13 hour period. Unlike the WT, the foci did not disperse after 3 hours in the $\Delta gshAB$ strain (Figure 3.9 B). By 10 hours the foci had still not dispersed. After 13 hours the foci were less detectable, however we also observed that this occurred around the time that the $\Delta gshAB$ strain began to exit lag phase (Figure 3.9 A & B). To investigate if the foci were truly dispersing or simply being diluted in the population of now actively growing cells, we resuspended N-24 bacteria in media containing only ammonium chloride, or both ammonium chloride and glucose with chloramphenicol. Chloramphenicol inhibits bacterial growth by binding to the 50S subunit of the bacterial ribosome, thereby halting protein synthesis. Both of these media would not support bacterial growth whilst supplying the cells with nitrogen to relieve nitrogen stress. We know from previous work that in WT cells, Hfq foci will disperse under both of these conditions⁸⁹. The $\Delta gshAB$ Hfq foci did not disperse upon relief of N starvation in either of these conditions, demonstrating that Hfq foci are not reversible in $\Delta gshAB$ *E. coli* (Figure 3.9 B). We observed an increase in lag time in $\Delta gshAB$ when compared to WT when bacteria were recovered in fresh N and C rich media at N-24 (13 hours vs 3 hours respectively) (Figure 3.9 A). The inability to efficiently recover from long term N starvation demonstrates glutathione is required for optimal adaptation to N starvation, as cells are greatly compromised upon relief of N stress at N-24.

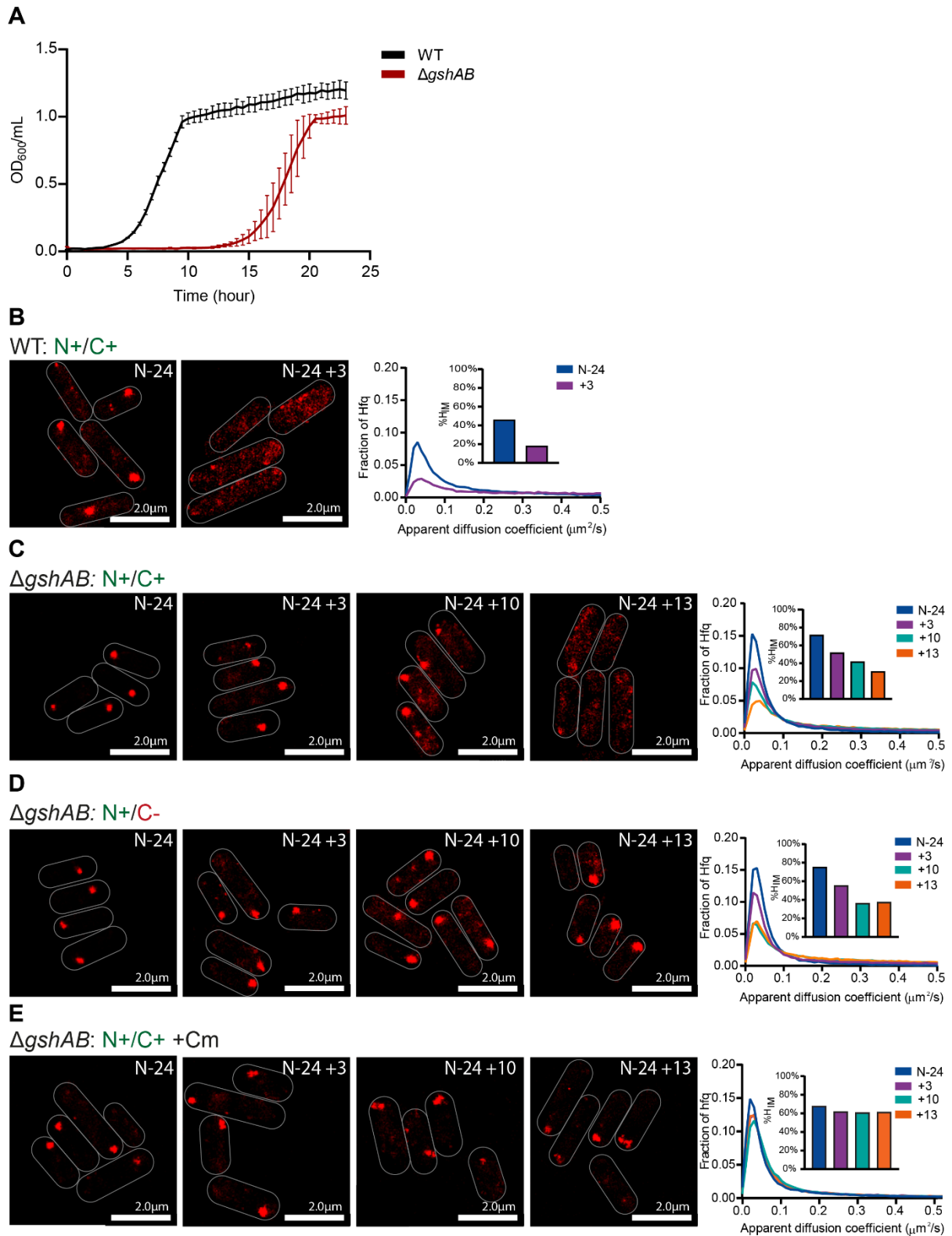


Figure 3.9: Hfq foci dispersal in $\Delta gshAB$ *E. coli*

(A) Long-term N starved (N-24) *E. coli* were resuspended in Gutnick media containing 10 mM ammonium chloride. Recovery growth of WT and $\Delta gshAB$ *E. coli* following resuspension was measured by OD600nm. (B-E) Representative PALM images of Hfq in WT and $\Delta gshAB$ *E. coli*. Bacteria were resuspended in fresh medium with different combinations of nitrogen and carbon containing (B) WT N+/C+; (C) $\Delta gshAB$ N+/C+; (D) $\Delta gshAB$ N+/C-; (E) N+/C+. Bacteria were imaged at N-24, then 3, 10 and 13 hours post resuspension. Graphs display the apparent diffusion coefficient of Hfq, inset bar graphs show %H_{IM}.

3.2.9. Glutathione is required for survival during long term N starvation

Previously, we observed no difference in initial growth or survival at N-24, between WT and $\Delta gshAB$ *E. coli* (Figure 3.5). We therefore concluded that the absence of glutathione did not affect growth or survival during N starvation. However, after observing a 10-hour increase in lag time following 24 hours of N starvation in the $\Delta gshAB$ mutant, relative to WT (shown in Figure 3.9 A), we reasoned that there may be differences in survival following longer periods of N starvation. Given that the mutant bacteria showed a substantially increased lag phase during recovery, it seemed likely that the cells may be severely compromised in some way by N-24. We therefore measured colony forming units at 24-hour intervals following N-24. We observed that survival of $\Delta gshAB$ was compromised following 48 hours of N starvation, and was further comprised following 72, and 96 hours (Figure 3.10 A). This demonstrates that glutathione is indeed required for survival long-term nitrogen starvation.

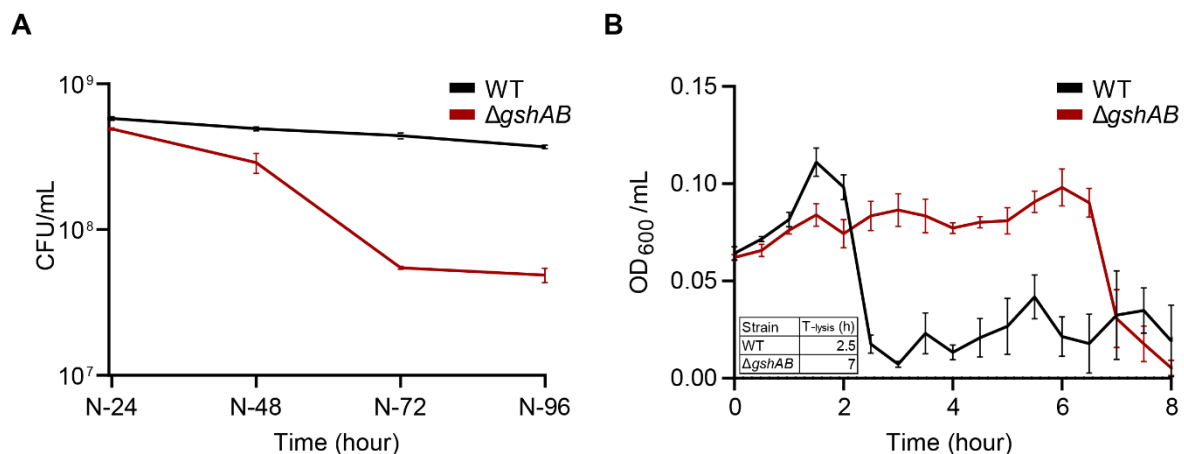


Figure 3.10: $\Delta gshAB$ survival is compromised after 48 hours of N starvation

(A) Viability of WT and $\Delta gshAB$ *E. coli* during long term N starvation measured by counting colony forming units. Cultures were sampled at 24-hour periods from N-24. (B) Lysis of bacteria by T7 phage was measured in WT and $\Delta gshAB$ *E. coli*. Graph showing the optical density OD₆₀₀ as a function of time following infection with T7 phage. The time taken for starting OD₆₀₀ to decrease by approximately 50% (T-lysis) are shown in the inset table. Error bars represent standard deviation (n=3).

Due to the survival defect observed in the $\Delta gshAB$ mutant, we reasoned $\Delta gshAB$ bacteria were likely existing in a metabolically different state to the WT strain. Previously, our lab has utilised a T7 phage assay to demonstrate differences in metabolism between *E. coli* strains⁸⁹. We used this assay as a surrogate to compare the metabolic state of the WT and $\Delta gshAB$ strains at N-24. We observed that the lysis time of $\Delta gshAB$ was increased compared to the

WT (7 and 2.5 hours respectively), indicating the metabolic state of the $\Delta gshAB$ was altered when compared to the WT. (Figure 3.10 B).

3.3. Hfq foci in $\Delta gshAB$ appear to form via the same canonical route as WT foci during N starvation

Having shown that the formation time and dispersal of Hfq foci in the $\Delta gshAB$ strain differ from those in WT *E. coli*, we considered whether differences in cellular physiology could lead to the observed Hfq foci being formed through a non-canonical pathway in $\Delta gshAB$. We therefore wanted to establish whether Hfq foci in the $\Delta gshAB$ strain were forming through a similar mechanism as the WT strain, with the formation dynamics simply being sped up rather than forming following a different trigger or through some other mechanism.

As the molecular trigger for Hfq foci formation is currently unknown, we compared known properties and features of Hfq foci formation between the WT and $\Delta gshAB$ strains. Based on what has been previously established, we focussed on the ability of foci to form independently of transcription and translation, whether the foci displayed properties of LLPS, and whether formation remained specific to N and not C starvation^{89, 99}.

3.3.1. Early Hfq foci formation during N starvation is independent of transcription and translation in $\Delta gshAB$ *E. coli*

As discussed during 1.3.2.3.2, when WT *E. coli* are treated with rifampicin or chloramphenicol after N run out, Hfq foci still form. This demonstrates that Hfq foci formation does not require *de novo* gene expression in WT *E. coli*⁸⁹.

To establish if Hfq foci formed independently of *de novo* gene expression in the $\Delta gshAB$ strain, we treated bacterial cultures with rifampicin or chloramphenicol shortly after entry into N starvation (N-). We then took PALM images at N-3 to determine whether Hfq foci continued to form early in the $\Delta gshAB$ mutant, as compared to the WT strain. We observed that Hfq foci still formed early by N-3 in the presence of both rifampicin and chloramphenicol, with %H_{IM} values similar to that of untreated $\Delta gshAB$ (Figure 3.11). We therefore concluded that the Hfq foci seen in $\Delta gshAB$ *E. coli* formed independently of both transcription and translation.

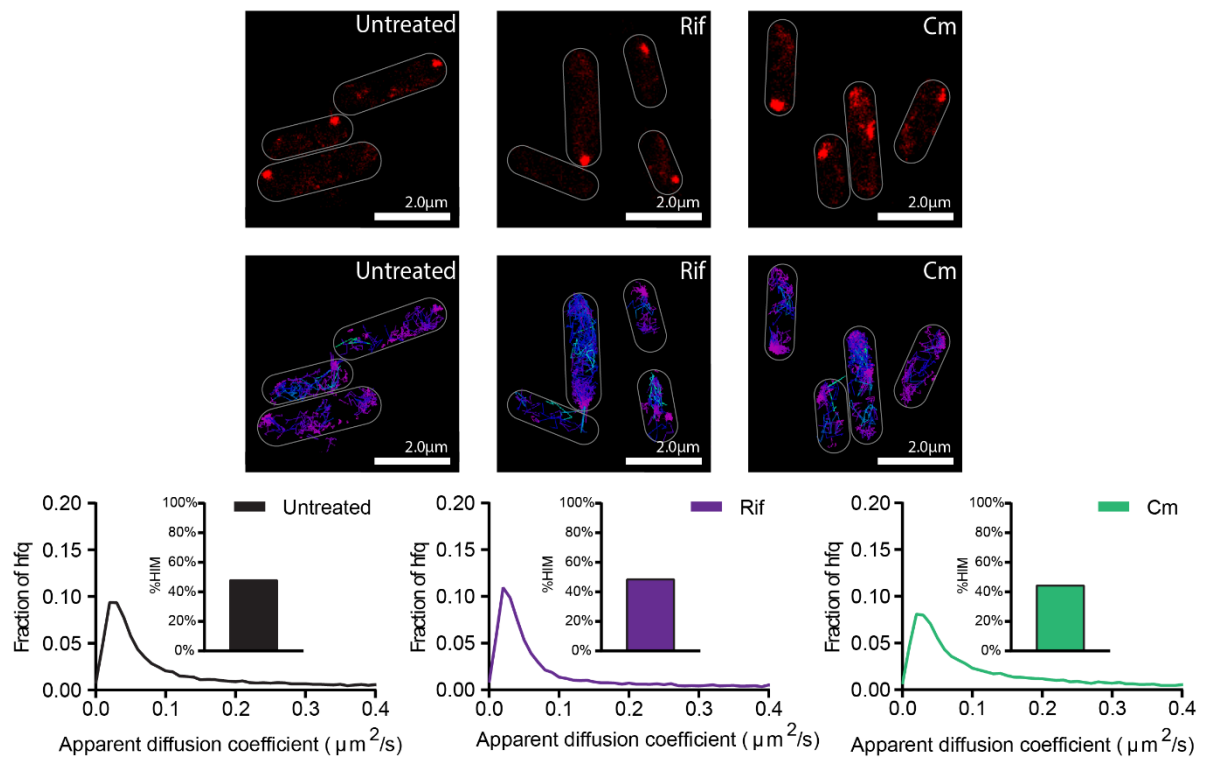


Figure 3.11: $\Delta gshAB$ Hfq foci form independently of *de novo* gene expression

Representative PALM images of Hfq in WT and $\Delta gshAB$ *E. coli* at N-3. Cultures were treated with 100 $\mu\text{g/mL}$ rifampicin or 150 $\mu\text{g/mL}$ chloramphenicol at N-. Graphs display the apparent diffusion coefficient of Hfq. The inset bar graphs show %H_{IM}.

3.3.2. Hfq foci in $\Delta gshAB$ display properties of liquid-liquid phase separation

A well-established test for the liquid-liquid phase separation of protein structures is structure dispersal upon addition of hexanediol. Hexanediol is an aliphatic alcohol which disrupts non-covalent bonds. Hexanediol therefore disrupts interactions between molecules which form between LLPS proteins, resulting in dispersal. When hexanediol is removed, the non-covalent bonds can re-form allowing the LLPS structure to reassemble. See introduction 1.3.1 for more details. Upon addition of 10% hexanediol, Hfq foci dispersed in both WT and $\Delta gshAB$ *E. coli* (Figure 3.12). When hexanediol was removed from the culture the Hfq foci re-formed in both strains, demonstrating that Hfq foci likely form via LLPS in $\Delta gshAB$.

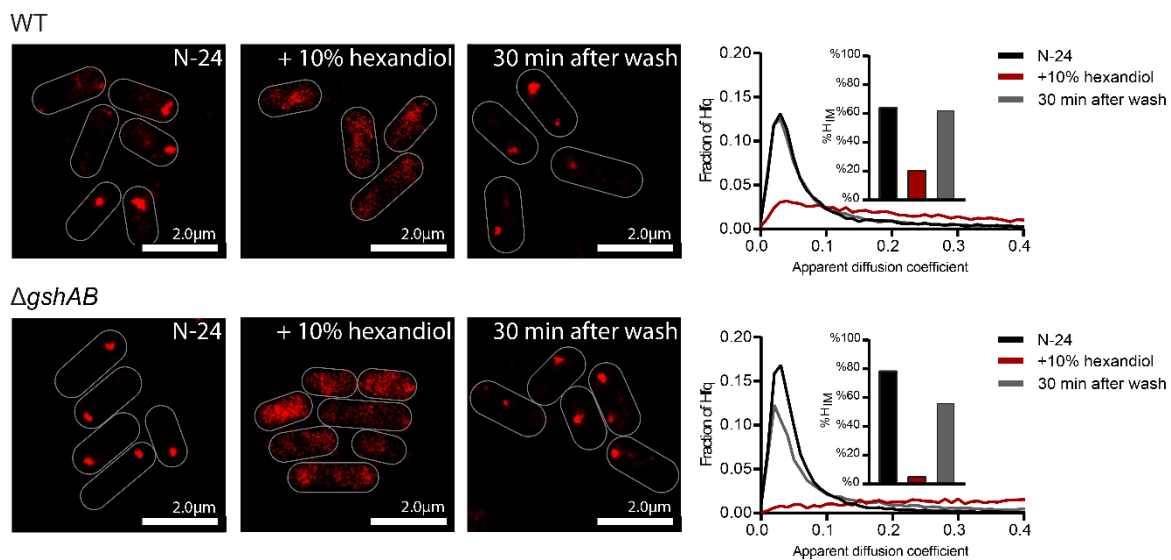


Figure 3.12: WT and $\Delta gshAB$ Hfq foci disperse upon addition of hexanediol

Representative PALM images of Hfq in WT (top panels) and $\Delta gshAB$ (bottom panels) *E. coli*. Cultures were imaged at N-24 (left) and treated with 10% w/v 1-6 hexanediol for 30 minutes¹¹. Cultures were reimaged 30 minutes after removal of Hexanediol (right). Graphs display the apparent diffusion coefficient of Hfq. The inset bar graphs show %HIM.

3.3.3. Hfq foci do not form in $\Delta gshAB$ *E. coli* during C starvation

We were interested in establishing if the trigger for $\Delta gshAB$ Hfq was likely the same as in WT bacteria. We reasoned if Hfq foci were forming via another route in $\Delta gshAB$ *E. coli*, we may observe them in a condition where they would not be observed in WT bacteria. As it has been shown that Hfq foci do not form when WT *E. coli* are starved for carbon (C)⁸⁹ we chose to use C starvation as a model to answer this question.

We used PALM microscopy to visualise $\Delta gshAB$ *E. coli* following 24 hours of C starvation. We saw no visible Hfq foci form in $\Delta gshAB$ *E. coli* under our C starvation conditions (Figure 3.13 A).

As well as determining the effect of glutathione on Hfq foci during N starvation, we also wished to establish if there is a link between the Hfq foci and bacterial survival. We have shown that the Hfq foci formation and dispersal is altered in $\Delta gshAB$, and that the survival of these bacteria is compromised during N starvation (Figure 3.10).

We did not observe Hfq foci formation in $\Delta gshAB$ bacteria during C starvation, we wished to establish if glutathione had any effect on long term survival during C starvation. We therefore starved $\Delta gshAB$ of carbon and observed cell survival. Long term survival during C starvation was not affected by the absence of glutathione in $\Delta gshAB$ *E. coli* (Figure 3.13 B).

In addition to compromised survival, $\Delta gshAB$ displayed an inability to recover from long term N starvation (Figure 3.9 A). We therefore also wished to characterise the ability of $\Delta gshAB$ to recover from long term C starvation. To do this, we resuspended C-24 bacteria in fresh Gutnick media containing 22 mM glucose. We saw a very modest difference in recovery dynamics from C-24 between WT and $\Delta gshAB$ *E. coli*, demonstrating that $\Delta gshAB$ were not compromised upon relief of C stress at C-24 (Figure 3.13 C).

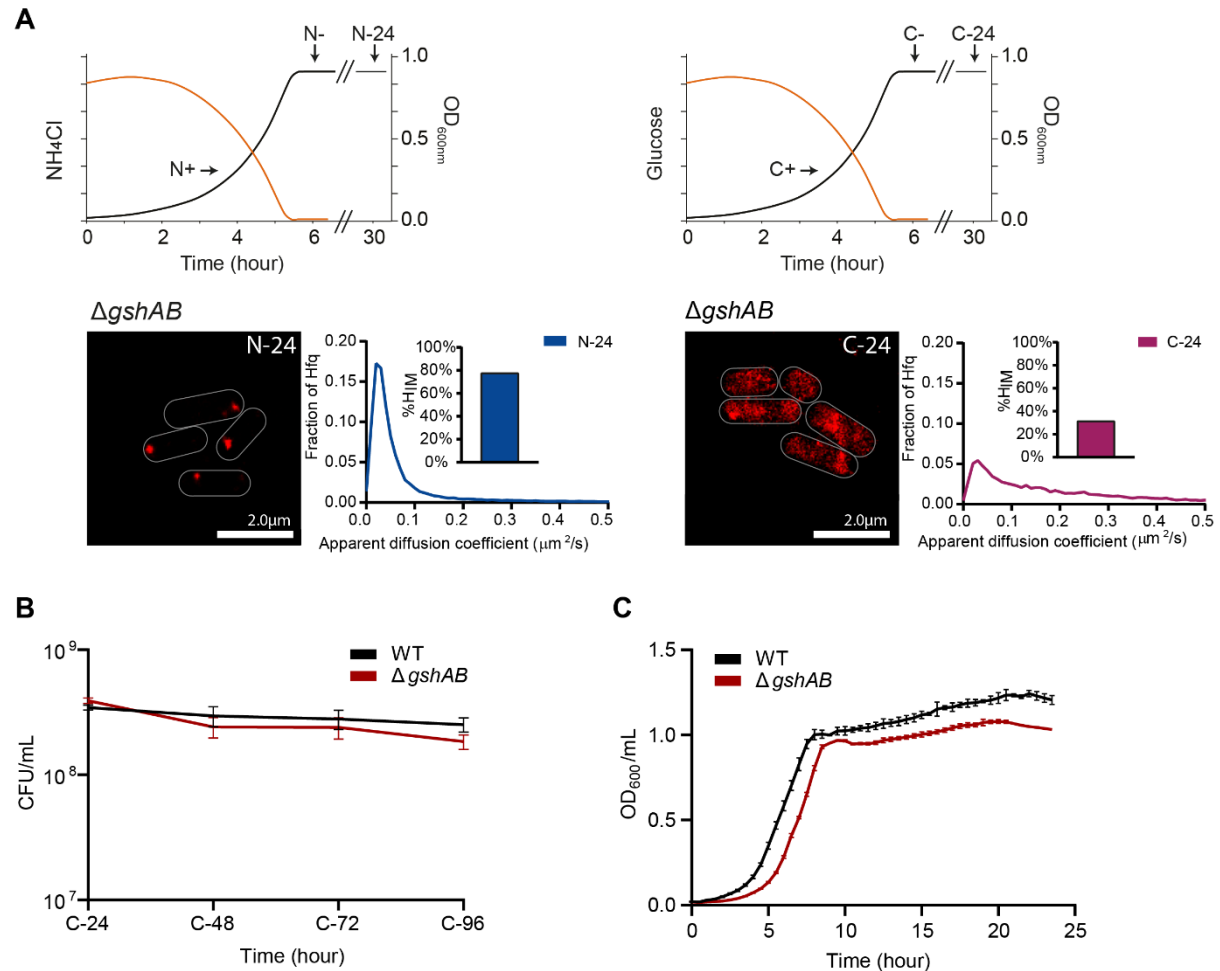


Figure 3.13: Hfq foci do not form under C starvation in $\Delta gshAB$ *E. coli*

(A) Representative PALM images of Hfq in $\Delta gshAB$ *E. coli* at N-24¹ and C-24 (C starved). Graphs display the apparent diffusion coefficient of Hfq. The inset bar graphs show %H_{IM}. (B) Viability of WT and $\Delta gshAB$ *E. coli* at N-24, measured by counting colony forming units. Error bars represent standard deviation (n=3). (C) Long-term C starved (C-24) *E. coli* were resuspended in Gutnick media containing 22 mM glucose. Recovery growth of WT and $\Delta gshAB$ *E. coli* following resuspension was measured by OD_{600nm}. Error bars represent standard deviation (n=3).

Together this data showed that Hfq foci formation, bacterial survival and recovery from stress were influenced by glutathione during N, but not C starvation. This provided us with a correlative link between the Hfq foci and cell survival, this link will not be investigated further in this work, however it will be discussed in more detail in Chapter 6.

3.3.4. Hfq foci do not form in the absence of TmaR in $\Delta gshAB$ *E. coli* during N starvation

TmaR has been shown in the literature to be necessary for Hfq foci formation under various conditions⁵². We considered whether the Hfq foci seen in $\Delta gshAB$ bacteria during N starvation remained TmaR dependent. We sampled $\Delta tmaR$ and $\Delta gshAB\Delta tmaR$ *E. coli* 24 hours into N starvation and imaged foci using PALM. We observed that the Hfq foci were unable to form by N-24 in either strain, showing that TmaR was necessary for foci formation under our conditions (Figure 3.14). This result further supported the hypothesis that Hfq foci were forming via the canonical route in $\Delta gshAB$ *E. coli*.

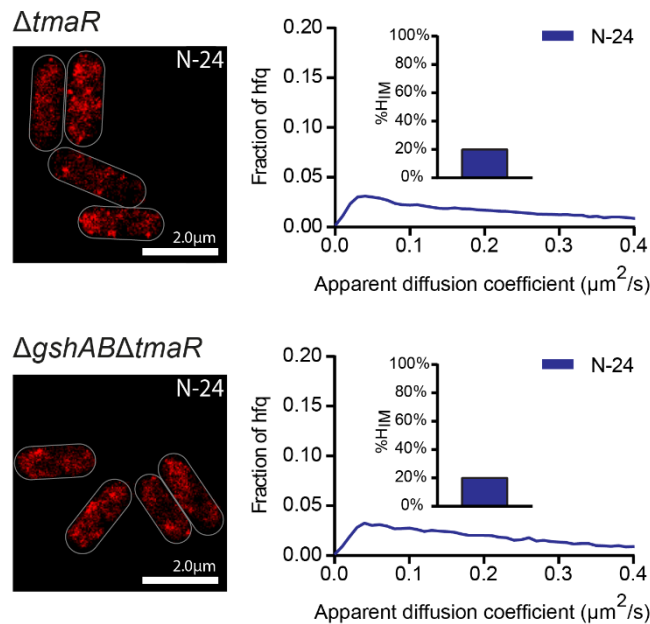


Figure 3.14: Hfq foci do not form in the absence of TmaR

Representative PALM images of Hfq in $\Delta tmaR$ and $\Delta tmaR\Delta gshAB$ *E. coli* after 24 hours of N starvation (N-24). Graphs display the apparent diffusion coefficient of Hfq, inset bar graphs show %H_{IM}.

Here we have demonstrated that in both the WT and $\Delta gshAB$ background, Hfq foci formed independently of *de novo* gene expression via liquid-liquid phase separation, in response to N but not C starvation, and this process was dependent on TmaR. This showed that many of the physical characteristics of the Hfq were still the same in both the WT and $\Delta gshAB$

backgrounds. Whilst we do not explicitly know the molecular trigger for foci formation, we nonetheless concluded that the foci seen in the $\Delta gshAB$ background were likely forming through the typical/canonical pathway, albeit with some likely differences due to other physiological changes in the cell.

We concluded that the data, albeit correlative, supported the hypothesis that the Hfq foci are forming specifically to some stress which occurred during N starvation. The ability to form these foci at the correct pace may affect long term survival and recovery from N starvation. Due to time constraints this hypothesis will not be investigated in this thesis, but will be followed up in future work by the group.

3.4. Exogenous glutathione changes the adaptive response to N starvation in $\Delta gshAB$ *E. coli*

3.4.1. Addition of exogenous glutathione prevents early foci formation in $\Delta gshAB$ *E. coli*

We wished to determine if early Hfq foci formation in $\Delta gshAB$ could be prevented upon reintroduction of glutathione.

We previously deleted genes $\Delta gshA$ and $\Delta gshB$ to create the double mutant $\Delta gshAB$, which was unable to produce glutathione. These two genes exist at different loci on the bacterial genome and the promoter of *gshA* is not currently known. Due to these technical constraints, we instead opted to chemically complement $\Delta gshAB$ with exogenous glutathione. Intracellular levels of glutathione have been reported between 1 and 10 mM in the literature¹³¹⁻¹³³, we therefore used 1mM glutathione to complement our bacteria.

We observed that the addition of 1mM exogenous glutathione at N+ prevented early foci formation in $\Delta gshAB$ bacteria. We saw no distinct foci by N-3 in cultures supplemented with 1mM glutathione, only beginning to see foci formation by N-6, similar to the WT (Figure 3.15).

Interestingly, whilst the addition of exogenous glutathione seemed to prevent the early formation of Hfq foci in the $\Delta gshAB$ mutant, it did not appear to have as substantial an effect on the foci formation dynamics in the WT bacteria under our conditions (Figure 3.15). We do not currently know why this is, but hypothesised the WT already maintained an optimum levels of glutathione to successfully manage certain stresses under these conditions, and addition of excess glutathione did not convey any further benefit. Due to time constraints, we did not attempt to investigate this observation further in this thesis.

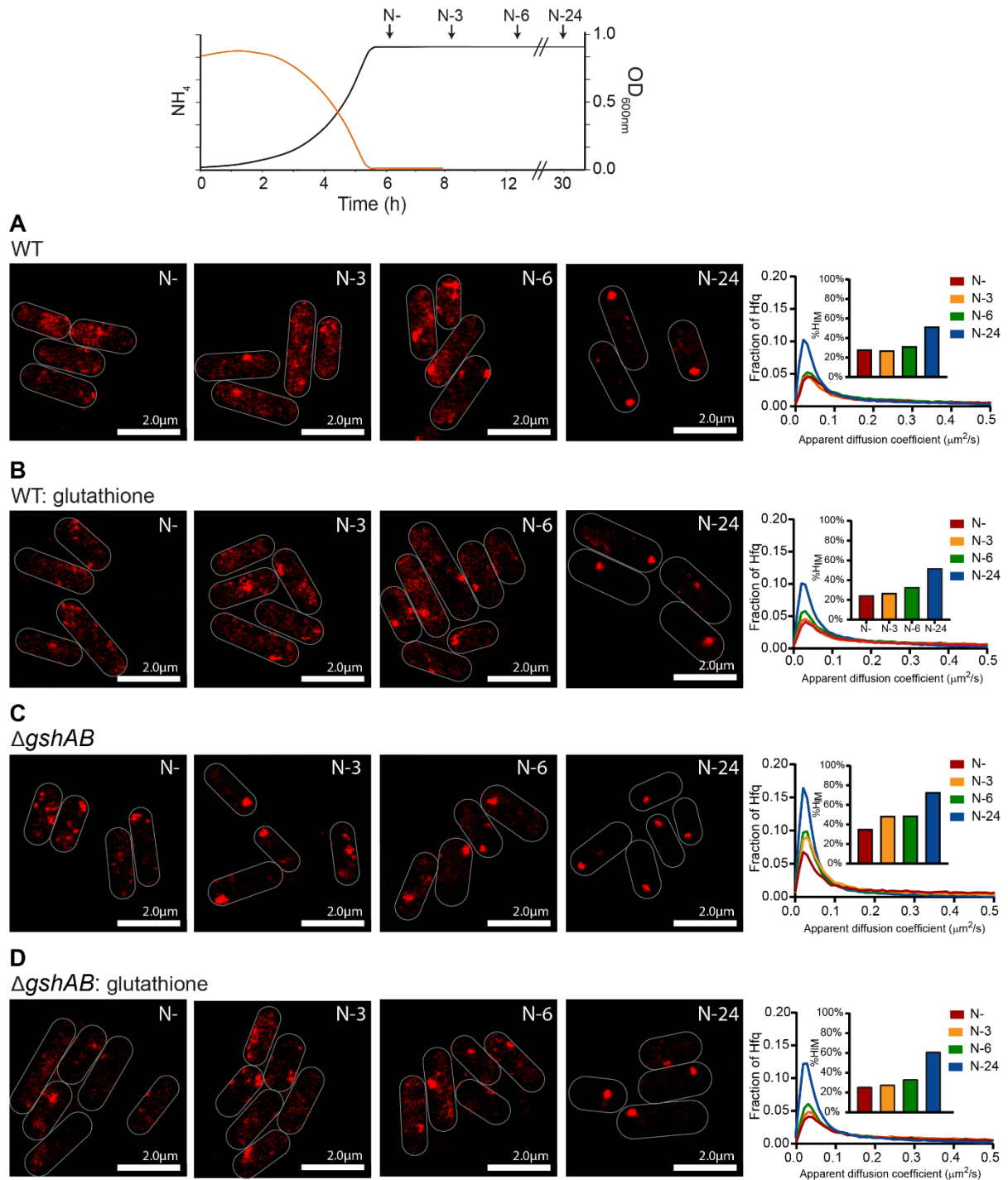


Figure 3.15: WT vs ΔgshAB foci formation in the presence of exogenous glutathione

Top graph indicates timepoints sampled for imaging. Representative PALM images of Hfq in WT and ΔgshAB *E. coli*. Bacteria were grown in Gutnick medium with 1mM glutathione added at N+ (A) WT; (B) WT + glutathione; (C) ΔgshAB ; (D) ΔgshAB + glutathione. Bacteria were imaged at indicated timepoints. Graphs display the apparent diffusion coefficient of Hfq, corresponding graphs show %H_{IM} in inset graphs.

3.4.2. Addition of exogenous glutathione delays entry into N starvation mode by acting as an alternative N source

Whilst conducting the experiments described in section 3.4.1, we measured the growth of WT and $\Delta gshAB$ bacteria in both treated and untreated cultures. Interestingly, both WT and $\Delta gshAB$ bacteria demonstrated diauxic growth when cultured with both ammonium chloride and 1mM glutathione (Figure 3.16). Both treated and untreated cells became growth attenuated as expected at approximately 5 hours once ammonium chloride had been depleted. After a brief pause in growth, cells treated with 1mM glutathione continued to grow, demonstrating that exogenous glutathione could be utilised as an alternative N source.

This observation led us to hypothesise that WT *E. coli* may use glutathione as an alternate N source during N starvation, thus delaying the formation of Hfq foci. Early foci formation observed in the $\Delta gshAB$ mutant may be triggered due to lack of glutathione availability as an alternative poor N source. We investigated this hypothesis further in chapter 4.

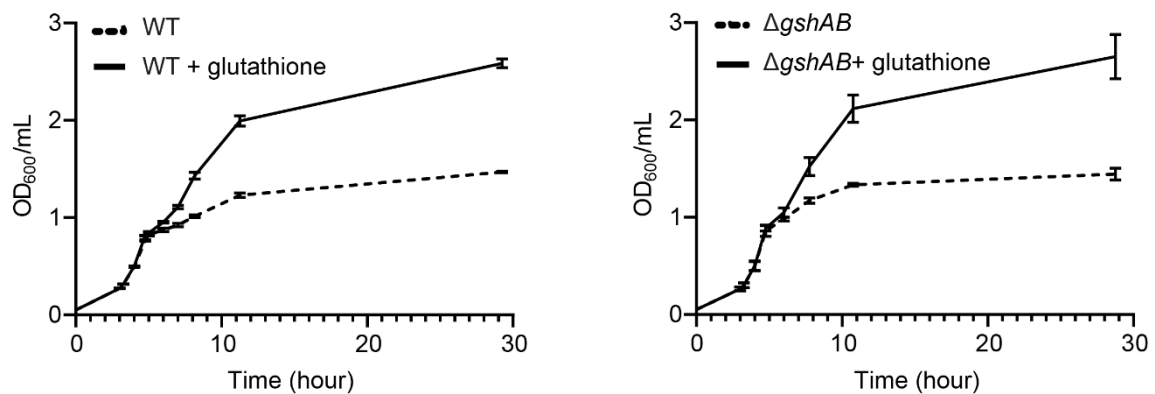


Figure 3.16: Entry into N starvation is delayed in cultures containing exogenous glutathione

Growth measured by OD₆₀₀nm of WT (left) and $\Delta gshAB$ (right) *E. coli* during N starvation. Cultures were treated with 1mM glutathione at N⁺. Error bars represent standard deviation (n=3).

3.5. Chapter summary

- Glutathione is upregulated during N starvation in *E. coli*
- Hfq foci form sooner in the absence of glutathione in $\Delta gshAB$ *E. coli*
- Hfq foci do not disperse upon relief of N starvation in $\Delta gshAB$ *E. coli*
- WT and $\Delta gshAB$ *E. coli* Hfq foci share similar characteristics suggesting that formation is governed by the same trigger
- Glutathione is required for long term survival and recovery from N starvation
- Glutathione can be used as an N source during N starvation

4. Chapter 4: Availability of glutathione as an alternate N source does not impact Hfq foci formation

4.1. Introduction

As shown in chapter 3, addition of exogenous glutathione to $\Delta gshAB$ *E. coli* prevents early foci formation, with foci formation dynamics returning to that of wild type bacteria. Whilst conducting these previous experiments, we measured growth of the bacteria in both treated and untreated cultures. We observed diauxic growth in bacterial cultures supplemented with glutathione, when compared to the control, suggesting that *E. coli* can utilise glutathione as an alternate N source. The prevention of early foci formation upon addition of exogenous glutathione coupled with *E. coli*'s ability to use glutathione as an alternate N source was particularly interesting to us, as the literature shows that Hfq foci formation in WT *E. coli* is observed during N starvation and is reversed upon alleviation of N stress ⁸⁹.

It could therefore be reasoned that *E. coli* may be utilising glutathione as an alternate N source under our N starvation conditions. This utilisation of an alternate N source may lessen N stress, thus delaying Hfq foci formation.

4.1.1. Breakdown of glutathione in *E. coli* is carried out by the enzyme gamma-glutamyl transpeptidase

Gamma-glutamyl transpeptidases¹⁸⁰ are highly conserved across a wide range of organisms, including mammals and bacteria. GGTs in prokaryotes have different roles depending on the organism¹²⁸. These roles range from breakdown of gamma-bond containing molecules to acting as virulence factors. In *E. coli*, GGT is the only peptidase enzyme known to break down glutathione. This key enzyme is a heterodimer consisting of a large and small subunit^{181 129}. GGT interacts with glutathione in the periplasm and catalyses hydrolysis of the hard to break gamma-glutamyl bonds of glutathione (Figure 4.1)¹⁸². This enzymatic reaction results in the GGT enzyme bound to a gamma-glutamyl residue and free L-cysteine-glycine. L-cysteine-glycine undergoes further dipeptidase cleavage and the products cysteine and glycine are transported into the cytoplasm^{129 136}. Here, a large proportion of cysteine is reincorporated into glutathione, the remaining pool being incorporated into the cell via protein synthesis¹³⁶.

Whilst much is known about the biochemical basis of the breakdown of glutathione by GGT

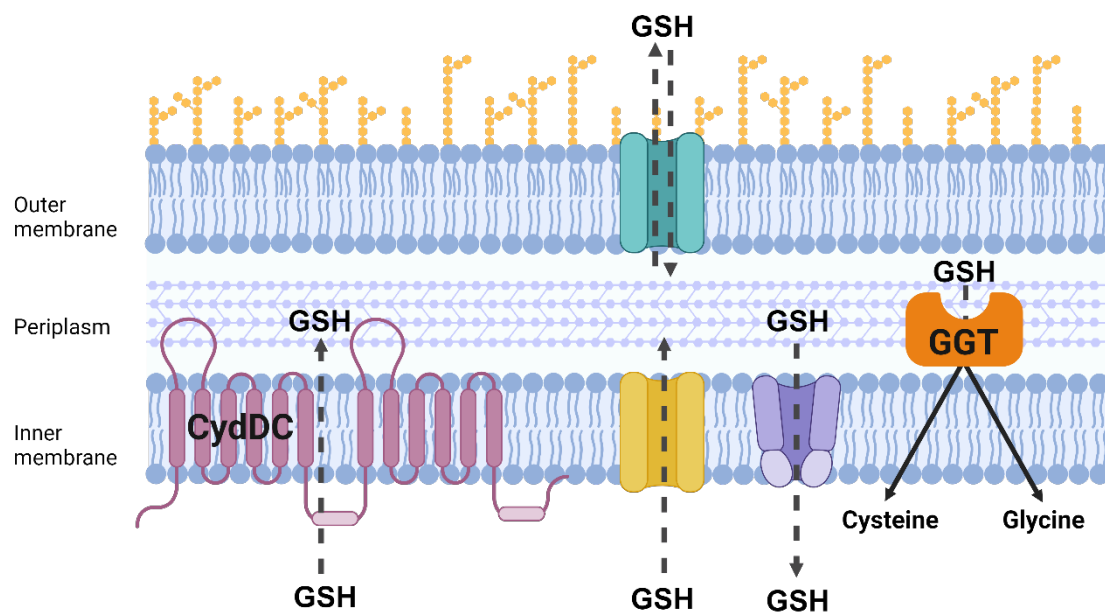


Figure 4.1: Gamma-glutamyl transpeptidase carries out enzymatic hydrolysis of glutathione in the periplasm

In *E. coli*, glutathione is transported from the cytoplasm to the periplasm by CydDC and other unknown transporters. Once in the periplasm glutathione is either exported through the outer membrane by outer membrane porins, or remains in the periplasm where it interacts with the enzyme gamma-glutamyl transpeptidase (GGT). GGT hydrolyses glutathione to the amino acids cysteine and glycine. These amino acids are then transported back to the cytoplasm. Glutathione can also be transported into the cell by an unknown transporter without undergoing degradation.

¹²⁹, little is known about the effect or purpose of this process on the homeostasis of glutathione

or amino acids in the cytoplasm. It has been shown that breakdown of glutathione by GGT can act as a cysteine salvage pathway during starvation conditions ^{136 180 183}. This literature supports our hypothesis that glutathione may be used as an alternate N source during N starvation, thus delaying the formation of the Hfq foci.

4.2. Chapter Aim

In chapter 3, we observed that *E. coli* can utilise exogenous glutathione as a N source during growth in N limited conditions. We hypothesised that *E. coli* may use glutathione as an alternate N source when preferred N sources (e.g., NH_4Cl) are depleted; thus, lessening N stress and delaying Hfq foci formation. Our aim was to determine whether the effect of glutathione on foci formation was due to utilisation of glutathione as an alternate N source or the protective properties of glutathione against an unknown cellular stress.

4.3. Availability of glutathione as an alternate N source does not impact Hfq foci formation

4.3.1. The amino acid components of glutathione are decreased at N-24 in ΔgshAB *E. coli*

As discussed (4.1), it has been demonstrated in the literature that glutathione can be scavenged as a source of both cysteine and glycine during starvation conditions ¹³⁶. To

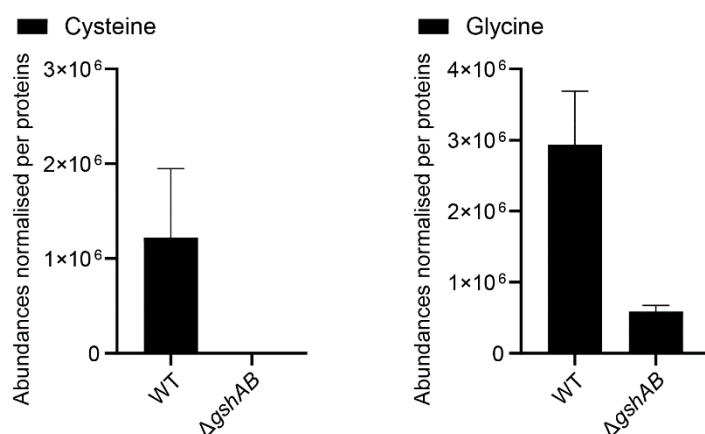


Figure 4.2 Cysteine and glycine levels are decreased at N-24 in ΔgshAB *E. coli* when compared to WT

Graphs show the abundances cysteine (left) and glycine (right) normalised per proteins. Metabolites were detected by targeted LC-MS metabolomics in both WT and ΔgshAB *E. coli* at N-24.

determine if there were differences in concentrations of either cystine or glycine between our WT and $\Delta gshAB$ strains during N starvation, we carried out targeted metabolomic analysis of both strains at N-24. We found both cysteine and glycine levels were substantially lower in $\Delta gshAB$ *E. coli* when compared to WT (Figure 4.2) at this timepoint. This strengthened our hypothesis that glutathione may be recycled as a N source by *E. coli* under our conditions. As Hfq foci form during N starvation, we reasoned the inability to produce and scavenge glutathione may be affecting the Hfq foci formation dynamics in $\Delta gshAB$ *E. coli*.

4.3.2. Addition of an alternate poor N source does not prevent early Hfq foci formation in $\Delta gshAB$ *E. coli*

We observed both diauxic growth and prevention of early foci formation in $\Delta gshAB$ *E. coli* cells cultures treated with exogenous glutathione. We therefore considered whether this prevention of early foci formation in $\Delta gshAB$ *E. coli* was simply due to glutathione being utilised as an N source. We therefore investigated whether addition of another, poor N source would have the same effect on growth and foci formation. Aspartate was chosen as it is one of the least favourable N sources of *E. coli*²⁵. Aspartate is a poor N source as it must first be broken down to ammonia before it can be assimilated by the cell. As shown (Figure 4.3), aspartate contains only one N whereas glutathione contains three, we therefore added three times higher concentration of aspartate than glutathione in order to provide the cells with comparable amounts of N.

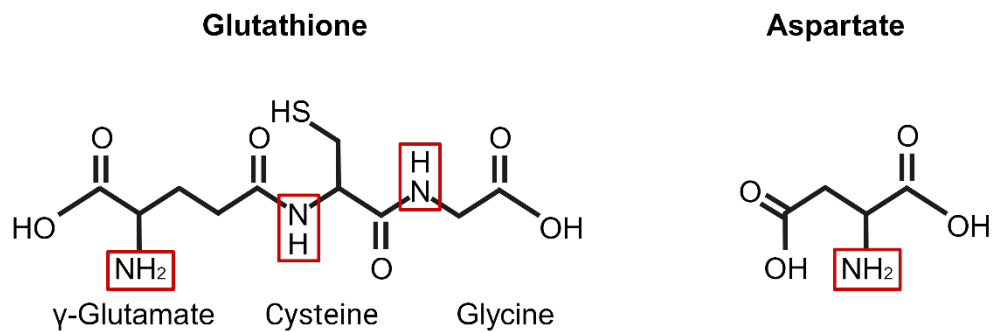
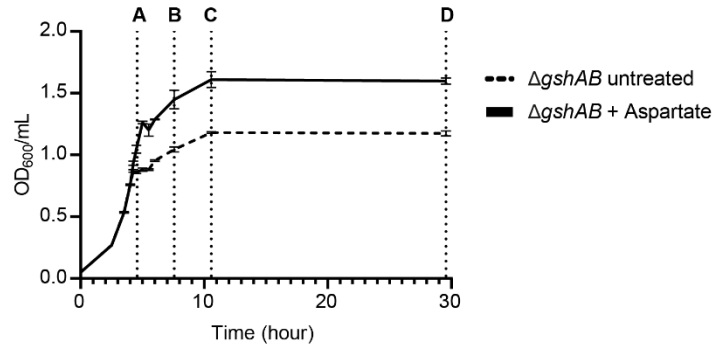


Figure 4.3: Glutathione and aspartate are N containing molecules

Glutathione consists of three N containing amino acids; γ -glutamate, cysteine and glycine. Aspartate contains only one N.

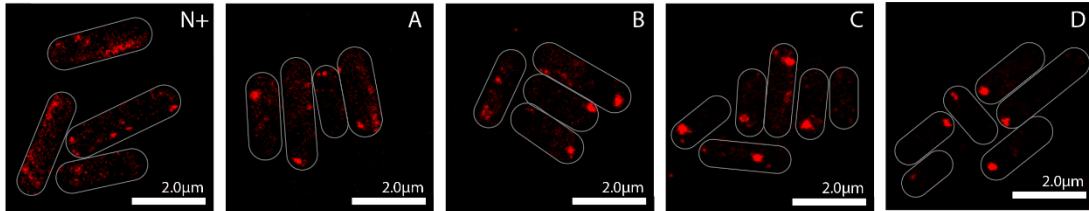
We imaged $\Delta gshAB$ cells experiencing N-starvation, supplementing cultures with additional aspartate at N+ (Figure 4.4). Compared to untreated cells, we observed that treatment with aspartate had no effect on early foci formation in $\Delta gshAB$ bacteria, with visible foci by N-3 (Figure 4.4 A & B). The %H_{IM} of both treated and untreated cells increased incrementally over time, as we have previously observed in this strain (Figure 4.4 C & D), with no notable differences between the two treatments. As with addition of glutathione, cells continued to grow when provided with an alternate, poor N source (aspartate) (Figure 4.4 A), demonstrating that in our condition the availability of an additional, poor N source does not necessarily prevent early foci formation. Given that addition of an alternate, poor N source did not affect the rate of foci formation in the $\Delta gshAB$ strain, we conclude that the prevention of early foci formation in $\Delta gshAB$ following addition of glutathione is somewhat specific to glutathione.

A



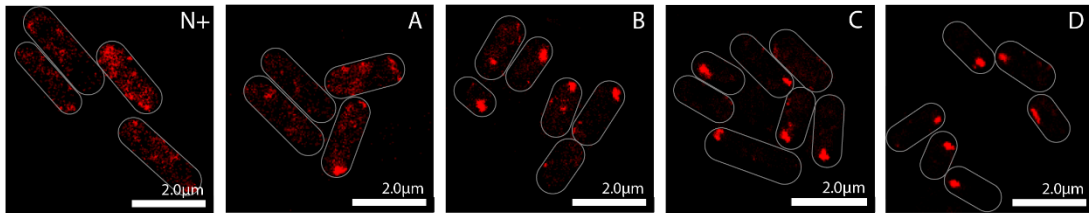
B

$\Delta gshAB$

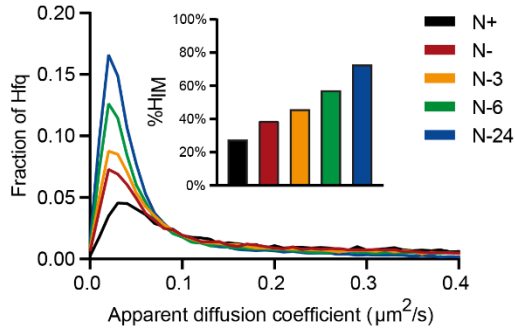


C

$\Delta gshAB$ + Aspartate



D



E

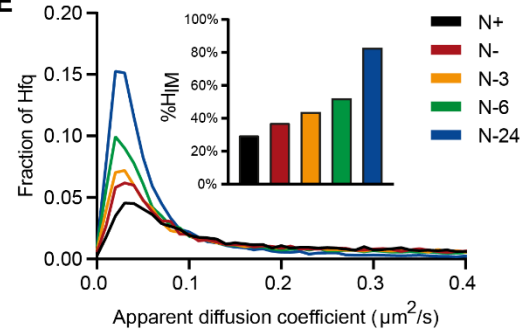


Figure 4.4: Early foci formation of Hfq foci in $\Delta gshAB$ *E. coli* is not prevented by addition of aspartate

(A) Growth measured by OD₆₀₀nm of WT and $\Delta gshAB$ *E. coli* during N starvation. For treated cultures, 3 mM aspartate was added at N+. (B-C) Representative PALM images of Hfq in *E. coli* (A) $\Delta gshAB$, (B) $\Delta gshAB$ + aspartate. Images taken at indicated timepoints during N starvation. (D-E) Graphs display the apparent diffusion coefficient of Hfq, inset bar graphs show corresponding %H_{IM} value (D) $\Delta gshAB$, (E) $\Delta gshAB$ + aspartate.

4.3.3. Breakdown of glutathione by GGT does not influence Hfq foci formation dynamics

To assess whether the breakdown of glutathione was necessary for preventing early foci formation in $\Delta gshAB$, we created a mutant strain lacking gamma-glutamyl transpeptidase (Δggt). Δggt was unable to break down glutathione into its constituent amino acids, therefore removing the ability of the bacteria to utilise glutathione as an alternate N source.

4.3.3.1. Deletion of GGT does not alter growth dynamics or survival during the first 24 hours of N starvation

To ensure that deletion of *ggt* did not affect growth dynamics or survival when compared to our background strains, we grew WT, Δggt , $\Delta gshAB$ and $\Delta gshAB\Delta ggt$ under our N starvation conditions. As shown in Figure 4.5, we observed no difference in initial growth dynamics between the strains. In addition, we measured cell survival at N-24, where all strains had similar viable cell counts, demonstrating that deletion of *ggt* had no effect on viability by N-24.

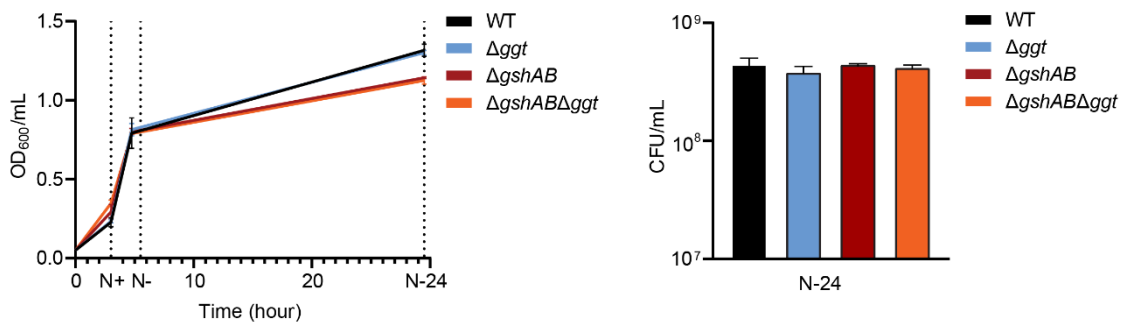


Figure 4.5: Initial growth and survival during N starvation is not compromised after deletion of *ggt* in *E. coli*

Growth measured by OD_{600nm} of WT, Δggt , $\Delta gshAB$ and $\Delta gshAB\Delta ggt$ *E. coli* during N starvation (left). Viability of all strains at N-24, measured by counting colony forming units (right). Error bars represent standard deviation (n=3).

4.3.3.2. Deletion of GGT does not influence Hfq foci formation dynamics

After confirming that deletion of *ggt* did not affect the initial growth dynamics or cell viability at N-24, we proceeded to characterise the Hfq foci in the Δggt strains. We saw no visual difference in Hfq foci formation dynamics between the WT and Δggt or between the $\Delta gshAB$ and $\Delta gshAB\Delta ggt$ strains (Figure 4.6); additionally, the apparent diffusion coefficient and %H_{IM} were similar between the parent and Δggt strains. This demonstrated that *E. coli* did not need

to hydrolyse glutathione into its N containing components in order to maintain foci formation dynamics. Previously we considered that *E. coli* may use glutathione production as a N sink for utilisation later in N starvation, when it could be broken down by GGT into usable cystine and glycine. Whilst this still may be true, it appears that this has no effect on foci formation during the timescale we observed. We predicted that deletion of *ggt* would also not affect foci formation dynamics in our $\Delta gshAB$ strain. As expected, in both the $\Delta gshAB$ and $\Delta gshAB\Delta ggt$ strains foci form by N-3 (Figure 4.6) as previously observed. The Hfq foci appear to be visually very similar by N-24, this similarity is reflected by %H_{IM} values.

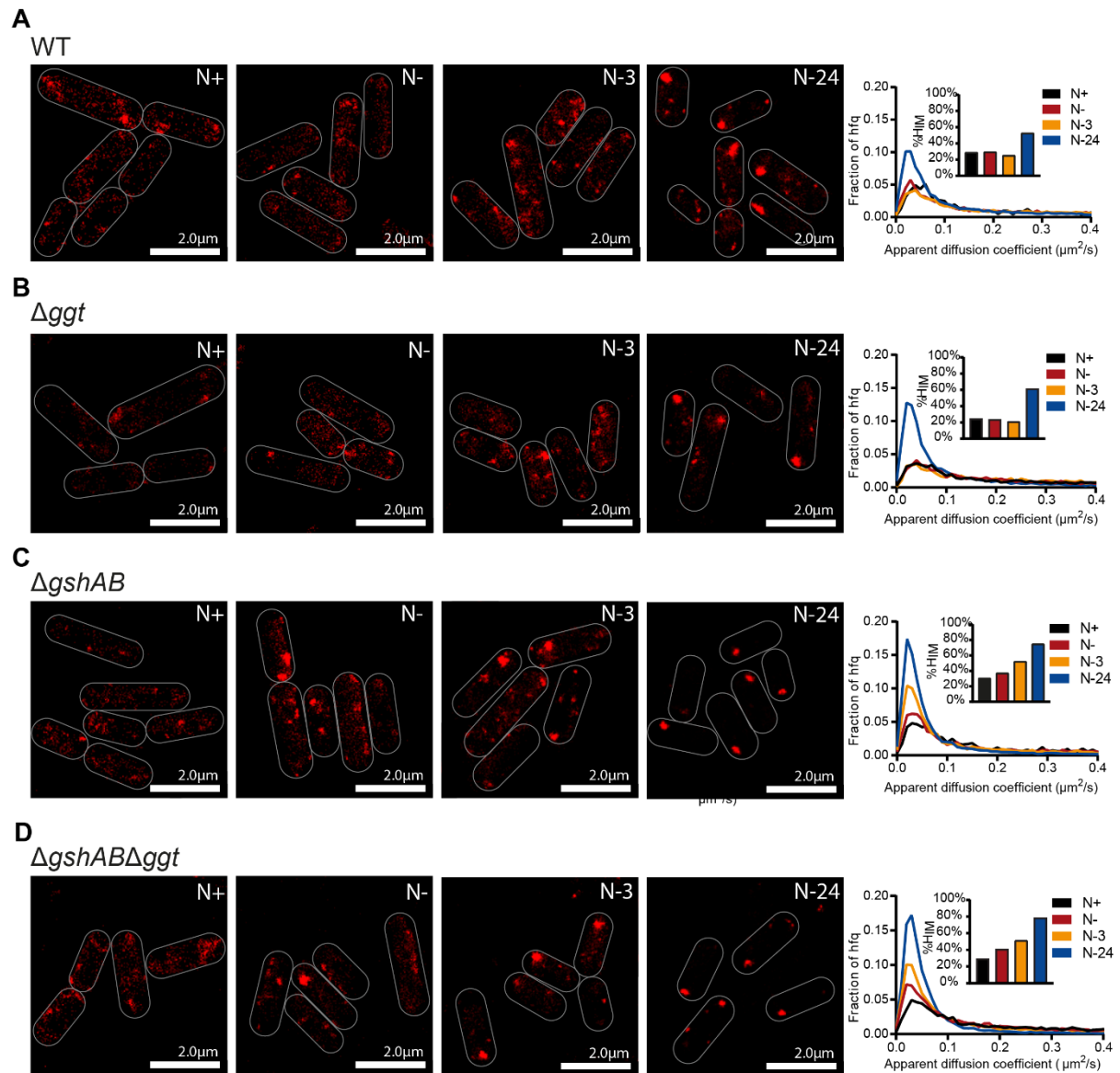


Figure 4.6: Deletion of *ggt* from WT and $\Delta gshAB$ *E. coli* does not affect Hfq foci formation during N starvation

(A-D) Representative PALM images of Hfq in *E. coli* during N starvation, timepoints indicated. Graphs show apparent diffusion coefficient of Hfq and %HIM values (inset). In (A) WT, (B) Δggt , (C) $\Delta gshAB$, (D) $\Delta gshAB\Delta ggt$

4.3.3.3. Exogenous glutathione prevents early Hfq foci formation in the absence of GGT

We wished to ascertain the role of exogenous glutathione in preventing early foci formation in *ΔgshAB E. coli*. We compared foci formation dynamics in cultures containing 1mM glutathione added at N+ to untreated controls in our strains of interest. We sampled cultures for imaging at timepoints A, B, C and D indicated on relevant graphs (Figure 4.7 A).

We observed cultures of *Δggt* and *ΔgshABΔggt E. coli* containing 1mM glutathione continued increase in OD₆₀₀ compared to their untreated control (Figure 4.7 A). This difference in OD₆₀₀ was not as great as that seen in WT and *ΔgshAB* treated vs untreated in chapter 3 (Figure 3.16), confirming that deletion of GGT impairs the ability of *E. coli* to use glutathione as an N source.

We note a small decrease in %H_{IM} in *Δggt* when compared to WT (Figure 4.7 C). Again, the effects of exogenous glutathione on WT bacteria did not appear to be as obvious as that of *ΔgshAB*. We hypothesise that there is an optimal amount of glutathione for the bacteria's survival during N starvation. Any excess glutathione may not be utilised to any further benefit in our conditions, or in the context of affecting foci formation. We will investigate this in future work.

Addition of 1mM exogenous glutathione to *Δggt* at N+ resulted in a decrease in %H_{IM} by N-6, showing that glutathione was able to somewhat delay foci formation in the WT background even when it could not be broken down by GGT (Figure 4.7, compare C and B). Interestingly, when we added glutathione to *ΔgshABΔggt* we saw a very visible prevention of early foci formation, relative to the untreated (Figure 4.7 E), with Hfq distribution, apparent diffusion coefficient and %H_{IM} values returning to values seen in the untreated WT control (Figure 4.7 B).

This data demonstrates that prevention of early foci formation following supplementation with glutathione does not result from providing the cells with an alternate N source, as glutathione does not have to be broken down by GGT in order to alter Hfq foci formation. In combination with the data shown in chapter 3, this data suggests that addition of glutathione likely prevents early foci formation by providing some other physiological protection against some other stress.

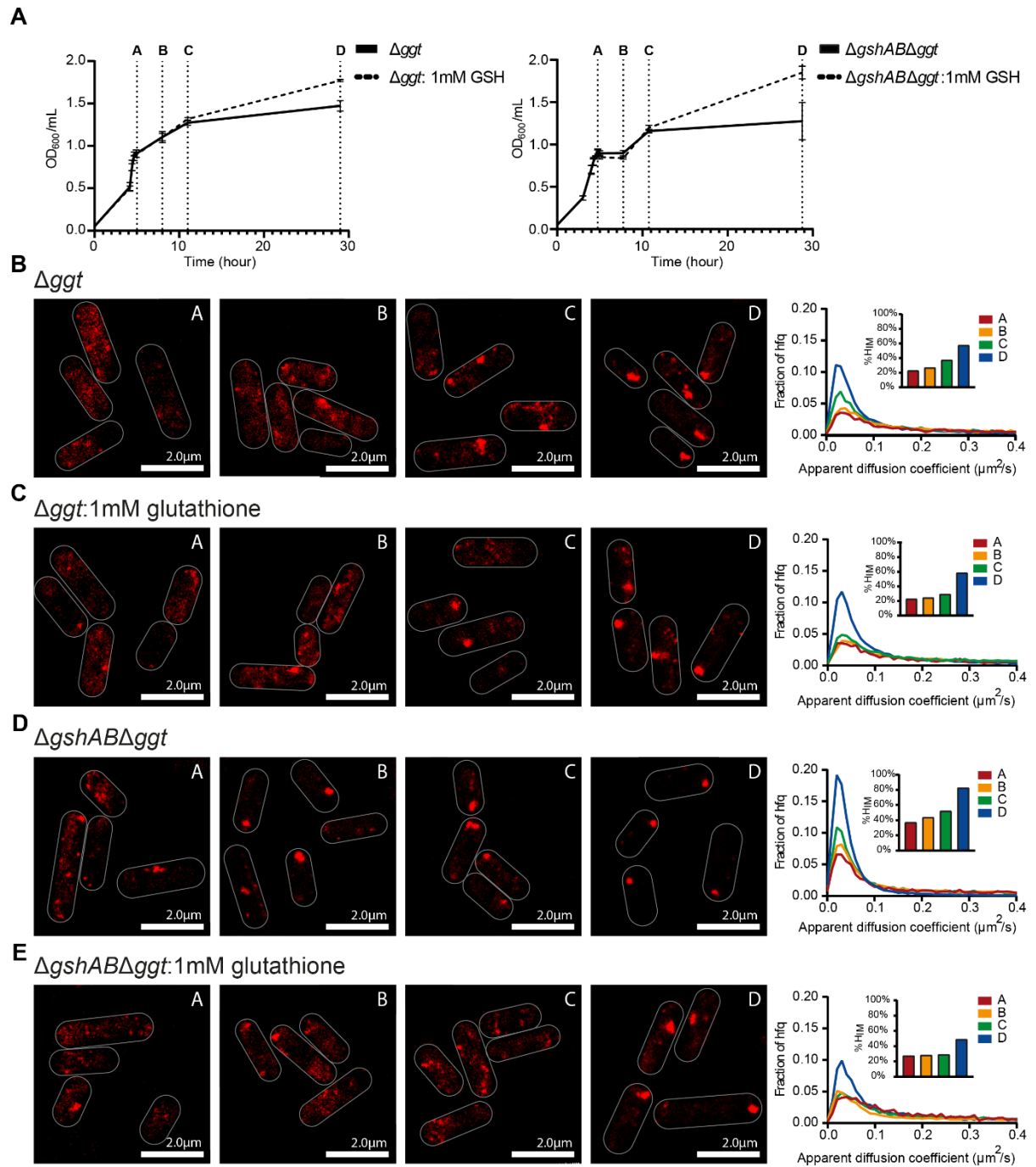


Figure 4.7: Early foci formation is delayed by addition of glutathione in Δggt *E. coli*

(A) Growth measured by OD_{600nm} of Δggt (left) and $\Delta gshAB\Delta ggt$ (right) *E. coli* during N starvation. Bacteria were grown in Gutnick medium with 1mM glutathione added at N+. (B-E) Representative PALM images of Hfq in *E. coli* in (A) Δggt , (B) Δggt + glutathione, (C) $\Delta gshAB\Delta ggt$, (D) $\Delta gshAB\Delta ggt$ +

4.3.4. Glutathione breakdown is not required for long term survival during or recovery growth dynamics following long-term N starvation

It has been shown previously that deletion of *ggt* often leads to an increased ability to withstand certain cellular stresses, as glutathione remains present in the cytoplasm at higher concentrations¹⁸⁴. We observed that deletion of *ggt* did not affect Hfq foci formation dynamics in WT *E. coli*, demonstrating that any increase in glutathione levels resulting from the absence of breakdown by GGT does not affect this particular response to stress. We therefore wanted to investigate if deletion of *ggt* influenced other phenotypic aspects of N starvation, such as long-term survival.

In many conditions glutathione is exported by *E. coli* during stationary phase^{136, 184}. There is no definitive explanation in the literature as to why bacteria export glutathione during particular phases of growth. It has been suggested that glutathione is exported to provide the cells with an exogenous source of amino acids at a later stage of growth, facilitating survival¹³⁶. We hypothesised that *E. coli* may use glutathione as a nutrient source to aid long term survival during N starvation.

Analysis of cell viability and recovery dynamics showed that deletion of *ggt* in both the WT and $\Delta gshAB$ background strains had no effect on long term survival during N starvation (Figure 4.8 A) or recovery in fresh media from N-24 (Figure 4.8 B). This data demonstrates that *E. coli* does not require the breakdown of glutathione by GGT for optimal survival during long term N starvation. We therefore conclude that the decrease in viability in the $\Delta gshAB$ strain during long term N starvation is due to some other cellular stress which is not tolerated in the absence of glutathione, and not because glutathione is unavailable as an alternative N source.

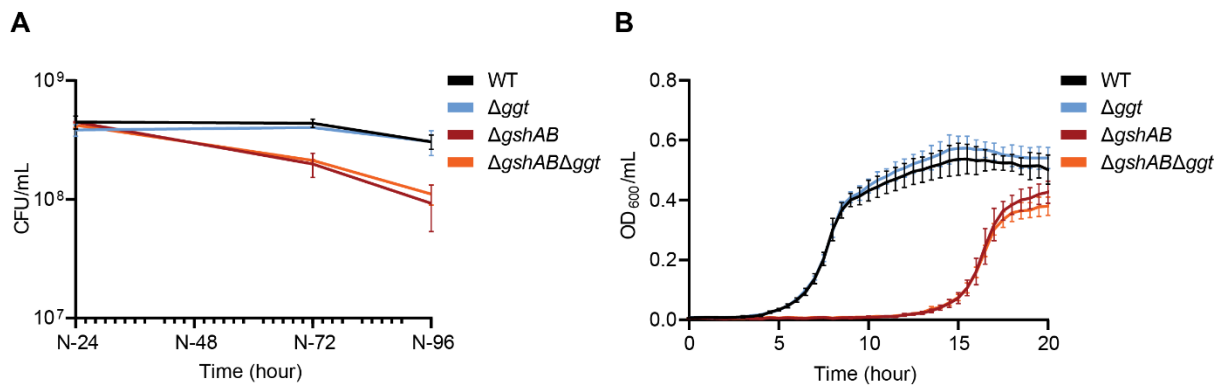


Figure 4.8: Deletion of *ggt* does not affect long term survival or recovery during N starvation

(A) Viability of WT, Δggt , $\Delta gshAB$ and $\Delta gshAB\Delta ggt$ *E. coli* from N-24, measured at 24 hour intervals by counting colony forming units. Error bars represent standard deviation (n=3) (B) Long-term N starved (N-24) *E. coli* were resuspended in Gutnick media containing 10 mM ammonium chloride. Recovery growth of WT, Δggt , $\Delta gshAB$ and $\Delta gshAB\Delta ggt$ *E. coli* following resuspension was measured by OD_{600nm}.

4.4. Chapter Summary

- Addition of an exogenous, poor N source does not prevent early foci formation in $\Delta gshAB$ *E. coli*, suggesting that glutathione availability as an N source does not govern foci formation dynamics.
- The ability of bacteria to hydrolyse glutathione does not affect foci formation in WT or $\Delta gshAB$ *E. coli*.
- Breakdown of glutathione by GGT is not required to prevent early foci formation in $\Delta gshAB$ *E. coli*.
- Breakdown of glutathione by GGT is not required for long term survival or recovery from N stress.
- Addition of exogenous glutathione restores the ability of $\Delta gshAB$ *E. coli* to tolerate some unknown cellular stress, therefore preventing early foci formation.

5. Chapter 5: Identification and characterisation of a $\Delta gshAB$ revertor mutant

5.1. Introduction

Study of liquid-liquid phase separated subcellular structures is an emerging field in microbiology. Whilst many structures have been identified (previously discussed in chapter 1), we are still a long way from assigning these structures specific physiological functions. One of the aims of this thesis was to investigate the function of the Hfq foci. Hfq is a ubiquitous protein, therefore deleting Hfq does not provide us with a useful tool to study the cell in the absence of the Hfq foci, due to the highly pleiotropic phenotypic effects of its absence. Whilst conducting the experiments in the previous chapters, we identified a mutant variant of $\Delta gshAB$ *E. coli* which no longer displayed compromised survival dynamics during long term N starvation. This mutant $\Delta gshAB$ provided us with a useful tool to study both survival and Hfq foci formation during N starvation.

5.2. Chapter Aim

We have hypothesised during this thesis that the Hfq foci may somehow be linked to survival during N starvation. Here, we aim to use our newly identified $\Delta gshAB$ revertor mutant, which displayed WT survival dynamics during N starvation, to determine if there was a link between survival and Hfq foci formation. We also aimed to identify the genomic mutations in this revertor and use these to elucidate the cause of survival defect in $\Delta gshAB$.

5.3. Identification and characterisation of a $\Delta gshAB$ revertor mutant

5.3.1. $\Delta gshAB$ revertor mutant survival is not compromised during long term N starvation

During previous N starvation survival assays, we identified $\Delta gshAB$ mutants which were able to survive long term starvation in a similar manner to WT *E. coli*. We have called these 'revertor' mutants, as their phenotype appeared to revert back to that observed in WT bacteria. To confirm the survival phenotype of the $\Delta gshAB$ revertor, we carried a long-term survival assay comparing all three strains. We saw the $\Delta gshAB$ revertor mutant survival dynamics mirrored that of the WT, whilst the $\Delta gshAB$ *E. coli* began to display compromised survival at N-72 (Figure 5.1).

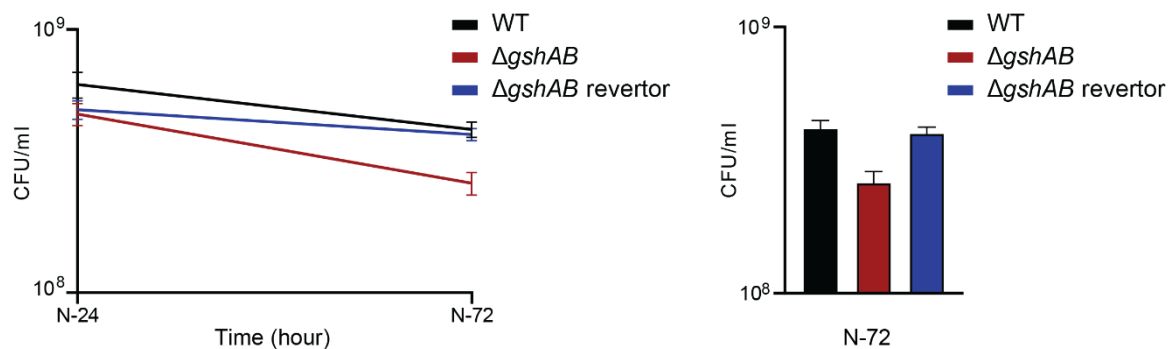


Figure 5.1: $\Delta gshAB$ revertor mutant maintains WT survival dynamics during N starvation

Viability of WT and $\Delta gshAB$ and $\Delta gshAB$ revertor *E. coli* from N-24, measured at 24-hour intervals by counting colony forming units (left). Viability of all strains at N-72 only (right).

5.3.2. Confirmation of $\Delta gshAB$ deletion in revertor strain

Before carrying out further experiments, we wished to confirm that *gshA* and *gshB* were still deleted in the $\Delta gshAB$ revertor mutant. We took single colonies of WT and $\Delta gshAB$ revertor and amplified both genes. Mutant deletion strains were previously made using kanamycin cassette via P1 phage transduction, therefore PCR were carried out using *gshA/B* forward and reverse primers, as well as a kanamycin specific reverse primer. We saw that both *gshA* and *gshB* genes were still successfully deleted in the $\Delta gshAB$ revertor (Figure 5.2).

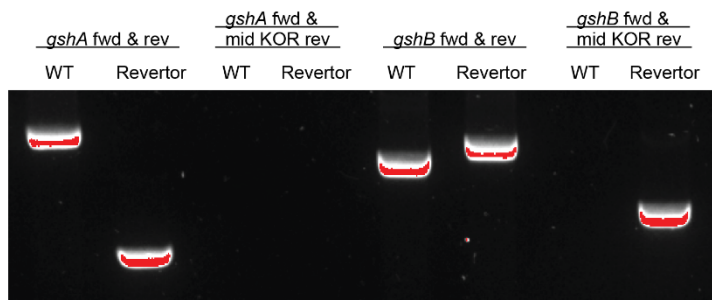


Figure 5.2: *gshA* and *gshB* are still deleted in revertor mutant

Colony PCR was used to confirm the presence of *gshA* and *gshB* genes in both WT and $\Delta gshAB$ revertor mutant. From left to right: amplification of *gshA* using flanking forward and reverse primers, amplification of *gshA* gene using flanking forward and kanamycin specific reverse primers (no band expected as strains were cured of kanamycin resistance at this loci), amplification of *gshB* using flanking forward and reverse primers, amplification of *gshB* gene using flanking forward and kanamycin specific reverse primers

5.3.3. $\Delta gshAB$ revertor mutation does not influences Hfq foci formation

As one of the aims of this work was to determine the function of the Hfq foci, we next wished to know if there was a correlative link between survival during N starvation and Hfq foci formation. To do this, we compared the foci formation dynamics of WT, $\Delta gshAB$ and the $\Delta gshAB$ revertor mutant.

We imaged WT, $\Delta gshAB$ and $\Delta gshAB$ revertor mutant cells experiencing different periods of N starvation. At N-, both $\Delta gshAB$ and $\Delta gshAB$ revertor showed the beginnings of Hfq foci formation (Figure 5.3 B and C), with small clustering of Hfq and higher %H_{IM} values than WT (Figure 5.3). Interestingly, the $\Delta gshAB$ revertor mutant still formed foci by N-3 mirroring the behaviour of Hfq in $\Delta gshAB$ (Figure 5.3 B and C).

Clearly, Hfq foci formation did not revert back to that of WT *E. coli* in the $\Delta gshAB$ revertor. We concluded that in this instance, survival of the $\Delta gshAB$ revertor did not correlate with Hfq foci formation.

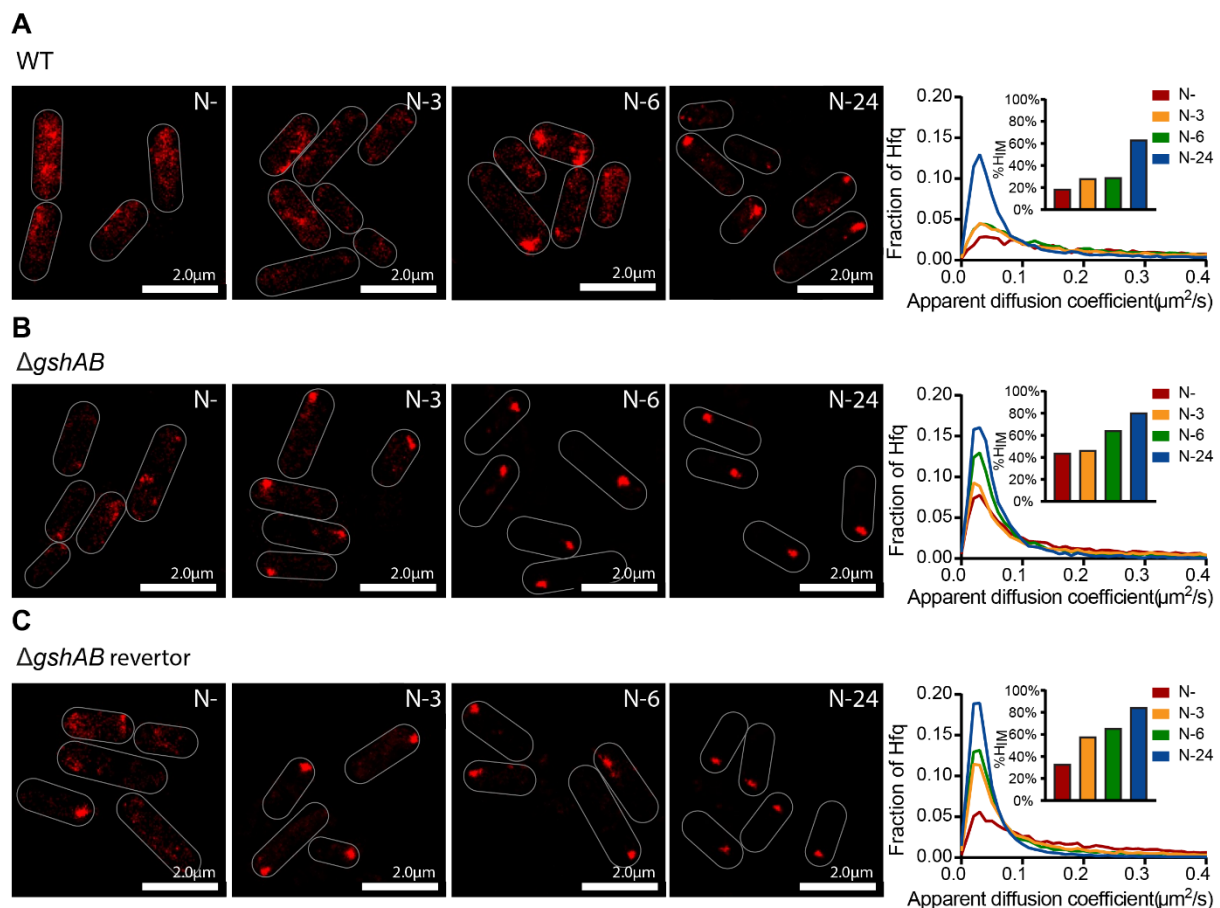


Figure 5.3: $\Delta gshAB$ revertor mutant foci formation dynamics are similar to that of $\Delta gshAB$

Representative PALM images of Hfq in WT and $\Delta gshAB$ *E. coli* in (A) WT, (B) $\Delta gshAB$, (C) $\Delta gshAB$ revertor. Bacteria were imaged at indicated timepoints. Graphs display the apparent diffusion coefficient of Hfq, corresponding graphs show %H_{IM} in inset graphs.

5.3.4. $\Delta gshAB$ revertor mutation influences Hfq foci dispersal

Previous work from our lab had established that an important feature of Hfq foci dynamics in *E. coli* is their ability to disperse upon relief of N stress (discussed in 1.3.2.3.2). In this work, we discovered (in section 3.2.8), that the Hfq foci in the $\Delta gshAB$ mutant did not disperse upon relief of N stress. Having determined that the $\Delta gshAB$ revertor retained $\Delta gshAB$ early foci formation dynamics, we next wished to determine if Hfq foci dispersal differed from $\Delta gshAB$. We observed that Hfq foci in WT are fully dispersed by +10 hours after relieving N stress (Figure 5.4 A). Hfq foci did not disperse upon relief of N stress by +13 hours in the $\Delta gshAB$ mutant (Figure 5.4 B). Unexpectedly, we observed that Hfq foci in the $\Delta gshAB$ revertor mutant had dispersed by +10 hours (Figure 5.4 C). We also observed a large drop in %H_{IM} in the $\Delta gshAB$ revertor after + 10 hours, with values in between that of WT and $\Delta gshAB$. As the

$\Delta gshAB$ revertor mutant showed an altered dispersal dynamic, we concluded there was a correlation between survival and Hfq foci dispersal.

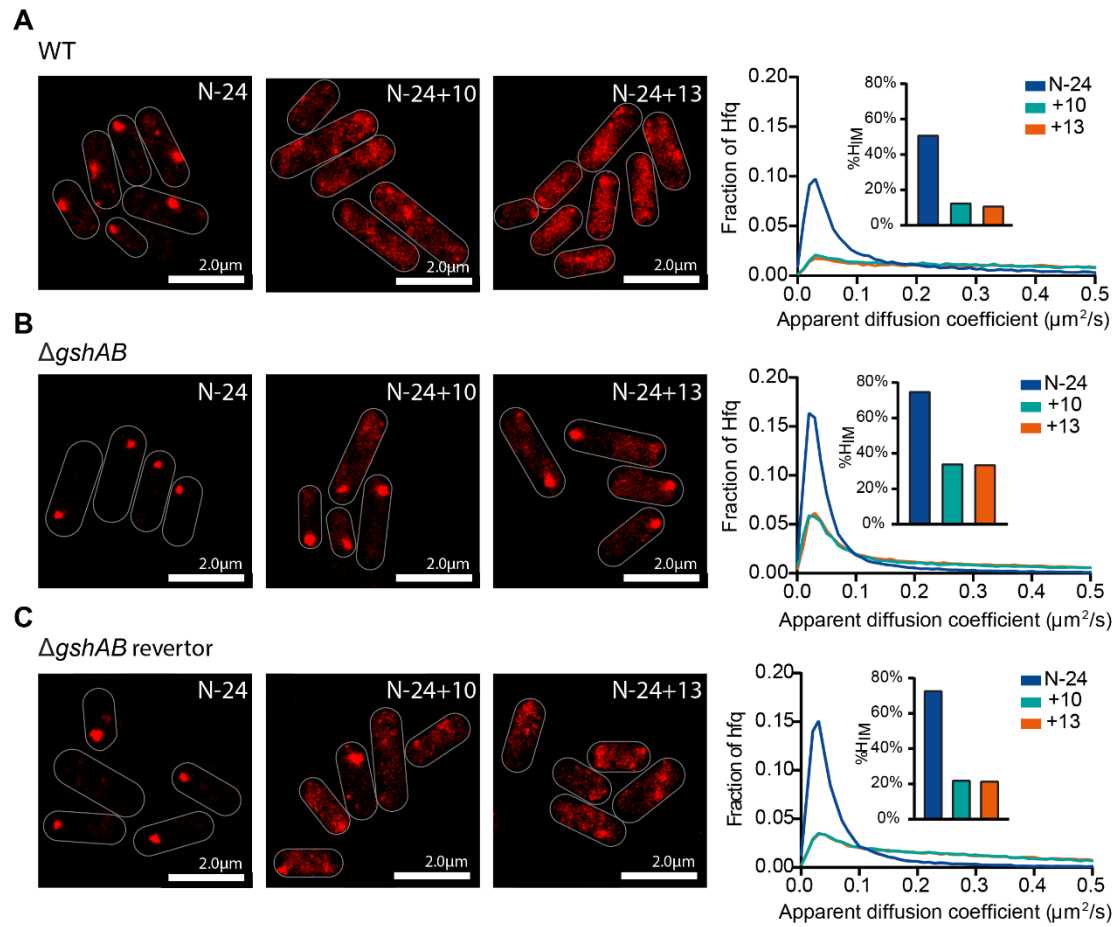


Figure 5.4: $\Delta gshAB$ revertor mutant foci dispersal dynamics differ from $\Delta gshAB$

(A-C) Representative PALM images of Hfq in N-24 *E. coli* (A) WT, (B) $\Delta gshAB$, (C) $\Delta gshAB$ revertor. Bacteria were resuspended in fresh medium containing 10 mM ammonium chloride and imaged at indicated timepoints. Graphs display the apparent diffusion coefficient of Hfq, inset bar graphs show %HfM.

5.3.5. $\Delta gshAB$ revertor mutation influences recovery growth dynamics following N starvation

We have shown in Chapter 3 that the recovery and survival of *E. coli* during N starvation correlated with the reversibility of Hfq foci when N starvation is alleviated. Interestingly, we observed that the $\Delta gshAB$ revertor strain Hfq foci dispersal and survival dynamics shifted to become more similar to those observed in WT during N starvation. This led us to consider whether the reversibility of the Hfq foci may be somehow linked with optimal recovery and survival of bacteria after and during long term N starvation.

To determine if there was a correlation between Hfq foci dispersal and lag time during recovery following N starvation, we compared recovery growth of the $\Delta gshAB$ revertor mutant to WT and $\Delta gshAB$ *E. coli*. As the $\Delta gshAB$ revertor displayed foci dispersal more similar to WT than $\Delta gshAB$, we predicted that the recovery time of this mutant would be similar to WT, rather than $\Delta gshAB$. Interestingly, we observed that the $\Delta gshAB$ revertor had an increased lag time and delayed recovery time when compared to both WT and $\Delta gshAB$ (Figure 5.5). This data suggests that reversibility of the Hfq foci was not directly correlated with recovery time. This was an unexpected result, which we will expand on further during the discussion.

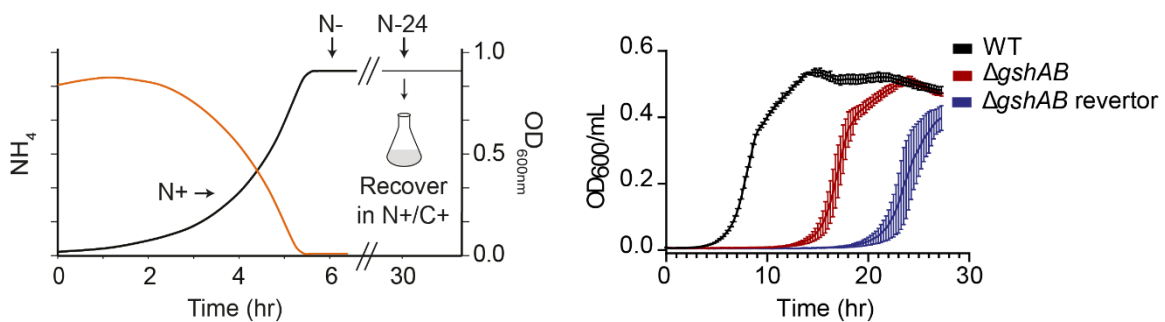


Figure 5.5: $\Delta gshAB$ revertor recovery growth is further compromised in comparison to $\Delta gshAB$

Schematic depicts N starvation model including timepoints sampled. Long-term N starved (N-24) *E. coli* were resuspended in Gutnick media containing 10 mM ammonium chloride. Recovery growth of WT, $\Delta gshAB$ and $\Delta gshAB$ revertor *E. coli* following resuspension was measured by OD_{600nm}.

5.3.6. Determining mutations in the $\Delta gshAB$ revertor

We observed some unexpected results in experiments carried out using the $\Delta gshAB$ revertor. To try and understand these results further, we wanted to know which mutations had occurred in the $\Delta gshAB$ revertor with the aim of identifying pathways involved in both survival and Hfq foci and dispersal.

Samples were taken by Dr Josh McQuail and sent for whole genome sequencing. We are currently awaiting sequencing results.

5.4. Chapter summary

- $\Delta gshAB$ revertor mutant displays WT survival dynamics during N starvation
- $\Delta gshAB$ revertor mutant displays $\Delta gshAB$ Hfq foci formation dynamics N starvation
- $\Delta gshAB$ revertor mutant Hfq foci dispersal dynamics shift closer to those seen in WT upon relief of N starvation
- $\Delta gshAB$ revertor mutant has an increased lag time during recovery from N starvation, when compared to both WT and $\Delta gshAB$

6. Chapter 6: Discussion

6.1. Introduction

During this work, we found that glutathione contributed to the adaptive response of *E. coli* during N starvation. Global metabolomic analysis showed an overall increase in glutathione levels during N starvation in *E. coli*. We observed that glutathione was upregulated during the early stages of N starvation and remained high throughout, and over 30% of the metabolites necessary for the glutathione biosynthesis pathway (cystine and γ -glutamylcysteine) were also enriched (Figure 3.2). To investigate the role of glutathione in *E. coli* during N starvation, we generated a $\Delta gshAB$ mutant which was unable to produce glutathione.

We demonstrated that glutathione was required for long term survival during N starvation (Figure 3.10) with $\Delta gshAB$ *E. coli* viability decreasing after 48 hours of starvation. We also observed a substantial increase in lag time in $\Delta gshAB$ *E. coli* when compared to WT during recovery in fresh N and C rich media at N-24 (13 hours vs 3 hours respectively) (Figure 3.9), demonstrating that metabolism in $\Delta gshAB$ *E. coli* was compromised during long term N starvation.

To determine a role for glutathione during N starvation, we utilised a subcellular structure associated with N stress, the Hfq foci. It has been shown that the Hfq foci form under certain stress conditions, including N starvation, osmotic and long term stationary phase^{52, 89}. The Hfq foci form gradually over time via LLPS, independently of *de novo* transcription and translation⁸⁹. Hfq foci formation dynamics are affected by the presence and binding of RNA as well as the PTS protein TmaR^{52, 89}. We found that the Hfq foci formed sooner in $\Delta gshAB$ *E. coli* during N starvation. Following 3 hours of N starvation (N-3), we observed distinct, dense foci forming in the $\Delta gshAB$ mutant bacteria not seen in the WT at the same timepoint. At N-3, the %H_{IM} had increased to approximately 45% in the $\Delta gshAB$ strain compared to 30% in the WT. By N-24, foci in $\Delta gshAB$ bacteria appeared denser and had a higher %H_{IM} value than the WT, showing that a larger proportion of Hfq was immobile in the foci within the mutant cells (Figure 3.6). Unlike the WT, we also observed that Hfq foci did not disperse upon relief of N starvation in $\Delta gshAB$ *E. coli* (Figure 3.9). Despite the difference in the timing of formation and altered dispersal dynamics, Hfq foci in WT and $\Delta gshAB$ *E. coli* still shared some similar biophysical characteristics (discussed in detail below), leading us to hypothesise that formation could be governed by the same cellular mechanism.

Interestingly, we observed that glutathione could be used an alternate N source during N starvation, observing diauxic growth when cultures were treated with 1mM exogenous glutathione. The addition of exogenous glutathione at N+ also prevented early foci formation in $\Delta gshAB$ bacteria. We saw no distinct foci form by N-3 in cultures supplemented with 1mM glutathione, only beginning to see foci formation by N-6, similar to the WT (Figure 3.15). To

determine if this was due to the utilisation of glutathione as an N source, we added an alternate N source at N+ and observed Hfq foci formation. Addition of exogenous aspartate (a similarly poor N source) did not prevent early foci formation in $\Delta gshAB$ *E. coli* (Figure 4.4). Additionally, the ability of *E. coli* to hydrolyse glutathione did not affect Hfq foci formation in WT or $\Delta gshAB$ (Figure 4.7), suggesting that glutathione availability as an N source did not govern foci formation dynamics. Addition of exogenous glutathione seemed to restore the ability of $\Delta gshAB$ *E. coli* to tolerate some unknown cellular stress, therefore preventing early Hfq foci formation. We also demonstrated that the breakdown of glutathione was not required for long term survival or recovery from N stress, suggesting that glutathione was not produced by *E. coli* to create an alternate N source sink which could be catabolised during later stages of N starvation.

We have shown that glutathione contributed to the adaptive response of *E. coli* during N starvation. Here, we will discuss how glutathione may be affecting bacterial physiology during N starvation and how this could be influencing the formation and dispersal of Hfq foci, as well as bacterial survival and recovery from N starvation.

6.2. Considerations when comparing the Hfq foci in $\Delta gshAB$, WT and other strains

This work demonstrated the Hfq foci form sooner in $\Delta gshAB$ mutant *E. coli* during N starvation when compared to WT (Figure 3.6). Hfq foci in WT *E. coli* typically began forming by N-6, becoming denser over time reaching a %H_{IM} of approximately 40 - 50% by N-24⁸⁹. In the $\Delta gshAB$ mutant Hfq foci formed sooner than in WT bacteria, reaching a %H_{IM} of 40% by N-3 (Figure 3.6). Additionally, Hfq foci were less able to disperse in $\Delta gshAB$ bacteria when compared to WT when N was replenished (Figure 3.9). We hypothesised that early Hfq foci formation and lack of dispersal could either be due to exacerbation of a physiological stress which also occurred in the WT, or that the Hfq foci were simply formed via a different mechanism in $\Delta gshAB$ bacteria and therefore might have displayed different properties. As we wanted to use these mutant bacteria to investigate Hfq foci formation, we needed to establish whether the foci observed in $\Delta gshAB$ and WT shared any properties which would affect their formation. This was an imperfect process, as the molecular trigger for the Hfq foci in WT bacteria during N starvation is unknown. We used the available literature to compile a list of properties possessed by Hfq foci in WT *E. coli*. The list of properties included: the ability of Hfq foci to form independently of *de novo* gene expression, dispersal upon hexanediol treatment (demonstrating the structure had properties of a LLPS condensate), formation under specific conditions and dependence upon TmaR. We then used these criteria to characterise

the Hfq foci in *ΔgshAB E. coli*. We observed that the *ΔgshAB* strain displayed similar properties to WT in these experiments. Hfq foci formation in *ΔgshAB* bacteria was not altered by the presence of either rifampicin or chloramphenicol, with foci appearing visually similar and %H_{IM} values remaining comparable to that of untreated *ΔgshAB* (Figure 3.11). We therefore concluded that the Hfq foci seen in *ΔgshAB E. coli* formed independently of both transcription and translation. Next, we wished to establish if Hfq foci in *ΔgshAB* displayed properties of a LLPS condensate structures. As previously discussed in 1.3.1, a well-established test for LLPS is dispersal upon addition of Hexanediol. Upon addition of 10% Hexanediol, Hfq foci dispersed in *ΔgshAB E. coli*. When Hexanediol was removed from the culture the Hfq foci re-formed, demonstrating that Hfq foci likely formed via LLPS in *ΔgshAB* bacteria (Figure 3.12). Hfq foci form only under specific conditions, including N but not C starvation. We saw no visible Hfq foci form in *ΔgshAB E. coli* under our C starvation conditions (Figure 3.13). We also observed that the Hfq foci were unable to form by N-24 in *ΔgshABΔtmaR E. coli*, showing that TmaR was necessary for foci formation in *ΔgshAB* during N starvation.

From these data, we concluded that Hfq foci in *ΔgshAB E. coli* are likely triggered by or formed in a similar way to those observed in WT. We will continue to investigate this in future studies and review as new information about the Hfq foci is published. Many of the aspects central to the physical formation of Hfq foci were the same in both WT and *ΔgshAB* bacteria. The absence of glutathione clearly had an impact on formation and dispersal, both of which are easily measurable allowing us to make direct comparisons with the WT. *ΔgshAB* is therefore a suitable and useful tool for the study of the Hfq foci.

6.3. How could glutathione affect LLPS of proteins?

As described above, it has been shown that Hfq foci (like many other subcellular structures) form via LLPS. Here, we consider how glutathione may affect LLPS of proteins.

6.3.1. Protein Concentration

It is well established that LLPS is influenced by the concentration of proteins ^{52 55}, therefore we first wished to rule out that Hfq foci weren't forming sooner in *ΔgshAB* bacteria simply due to a higher concentration of Hfq. We established that Hfq levels in *ΔgshAB* and WT bacteria were comparable at multiple stages of N starvation, therefore early formation of Hfq foci in *ΔgshAB* was unlikely due to protein concentration (Figure 3.7).

6.3.2. Viscosity and molecular crowding

We discussed in 3.2.2.3 that glutathione has a wide range of protectant properties against multiple stresses experienced by bacterial cells. Many of these protectant properties have consequences for the general biochemical state of the cell, such as viscosity and molecular crowding. Changes in viscosity can occur in response to stresses such as osmotic and nutrient deprivation¹⁸⁵. It has been demonstrated that the internal cellular state of *E. coli* changes when the metabolism is less active and cellular energy is depleted¹⁸⁶ - both of which occur during N starvation when cellular nutrients are depleted and cells enter a state of growth attenuation. Metabolic changes such as these can lead to a change in viscosity, which changes the ability of molecules such as nucleic acids and proteins to move about the cell¹⁸⁵¹⁸⁶. Changes in viscosity can alter the dynamics of LLPS, however this is heavily dependent on the condition and size of the LLPS droplets when viscosity changes¹⁸⁷. Interestingly, molecular crowding has been shown to increase LLPS of Hfq *in vitro*⁵², we therefore reasoned that viscosity and metabolic crowding may play a role in the movement and localisation of Hfq during N starvation *in vivo*.

To determine if viscosity and molecular crowding were changed in $\Delta gshAB$ bacteria during N starvation, we attempted to utilise μ NS dots¹⁸⁶. It is difficult to study the basal rate of diffusion of proteins within the cell, as most native proteins interact with other cellular components, thereby affecting their movement¹⁸⁸. We used an avian reovirus protein μ NS fused with GFP, a useful tool as it is completely foreign to the bacterial cell therefore less likely to make specific interactions. The protein self-assembles even when fused to GFP and can be expressed internally from a plasmid, eliminating the need to introduce non-biological probes into the bacteria¹⁸⁶. We expressed the μ NS dots, and attempted to quantify their movement in both WT and $\Delta gshAB$ bacteria at various timepoints during N starvation. However, N starvation proved a difficult condition to study using this approach, as N starved cells scavenged N from whatever source they can. Unfortunately, μ NS dots expressed during the early stages of growth did not continue to be visible by N-24, suggesting that they were not maintained sufficiently and were likely degraded. Future work to study the viscosity of the cytoplasm will have to consider these constraints. We did, however, hypothesise that other LLPS protein behaviours may be affected if glutathione causes changes in cellular viscosity or molecular crowding. We therefore attempted to determine if our observed phenotype was Hfq specific, or if other proteins also behaved differently in the absence of glutathione.

6.3.3. Hfq specificity

We wished to determine if our observations were specific to Hfq, or if the behaviour of other proteins which are known to undergo LLPS were also affected by the absence of glutathione.

We selected two proteins known to form LLPS structures; RpoC and RNase E^{85, 98}. Both RpoC and RNase E formed small protein clusters during all stages of N starvation, and we saw no difference in behaviour of either protein when comparing WT and $\Delta gshAB$ *E. coli* (Figure 3.8). From this data, we concluded that the behaviour of Hfq seen in $\Delta gshAB$ is somewhat specific to Hfq. Whilst this data is limited by the number of proteins studied, it is impractical to study an extensive array of proteins for behavioural changes in the absence of glutathione. This data was sufficient to conclude the effect of glutathione on Hfq seemed to be somewhat specific and allowed us to direct further study towards other avenues of enquiry.

This specificity to Hfq suggests that changes to LLPS dynamics were more likely due to factors other than viscosity or molecular crowding, such as post translational modifications⁶¹.

6.3.4. Post translational modifications

It is widely established that post translational modification of proteins causes changes in protein structure and function⁶¹. PTMs can occur during the natural progression of protein maturation, or as a response to stress conditions²². PTMs can also affect the process of LLPS, as PTMs change the biophysical properties of proteins such as charge, structure and hydrophobicity, which in turn changes how they interact and form intermolecular interactions⁶¹.

It is possible that Hfq itself, or proteins associated with Hfq, are post translationally modified during N starvation. Common PTMs include phosphorylation, glycosylation, methylation, Ubiquitination, S-Nitrosylation, N-Acetylation and carbonylation (of ketones & aldehydes). Some PTMs have been shown to occur in response to particular stresses, such as S-Nitrosylation and nitration which occur during nitrosative and oxidative stress respectively, where these PTMs can act as major signalling mechanisms⁶¹.

Phosphorylation acts as a quick way to regulate proteins in response to signals, and provides extensive regulation of enzyme activity in *E. coli*⁶². Phosphorylation is catalysed by protein kinases which create covalent bonds between phosphate group of ATP and the hydroxyl groups on amino acids. As phosphate groups are negatively charged, phosphorylation introduces a negative charge to proteins, altering their electrostatic charge and therefore their interactions along their polypeptide backbone⁶³. LLPS can be regulated by phosphorylation and can both enhance and inhibit the separation of proteins into phases⁶⁶. Acetylation can also change the electrostatic charge of proteins, changing positively charged residues to neutral – this process has been shown to regulate stress granule formation in eukaryotes *in vivo*⁵⁷. PTMs can influence LLPS by altering the hydrogen bonding capacity of a protein, rather than by altering the net charge. An example of this is arginine methylation, which increases hydrophobicity and consequentially the behaviour of the protein⁶⁷. The likelihood of

specific PTMs depends on the conditions faced by bacteria and the structure of a protein, therefore it is difficult to speculate which PTM is more likely under our condition.

Interestingly, glutathione itself can post-translationally modify proteins. Glutathionylation is a PTM where GSH binds to cysteine residues which have been exposed to reactive oxygen species¹⁴¹. Glutathionylation is a reversible reaction which protects proteins from permanent damage¹⁴¹. Although Hfq contains no cysteines and therefore cannot be glutathionylated, other proteins which co-localise with Hfq foci do. One such protein is RNase E; if modified, it is reasonable to speculate that this may affect its interaction with Hfq, nucleic acids or other proteins involved in Hfq foci formation during N starvation. In this thesis we compared the behaviour of RNase E in $\Delta gshAB$ and WT bacteria. We found no difference in RNase E behaviour, with RNase E appearing visually similar and having comparable apparent diffusion coefficient at most timepoints. This suggested that any direct PTM of RNase E did not change its ability to undergo LLPS under our conditions. However, this does not mean PTM of RNase E is not occurring, or that it does not affect Hfq foci formation as there may be a more indirect route of action.

The formation of condensed phases during LLPS is often reversible, however LLPS proteins can form gels when components of the dense phase cross link with each other and become physical gels^{72 73}. These gels often require high salt or denaturation to dissolve⁷⁴. Aggregation is said to have occurred when irreversible molecular interactions are formed and proteins enter a higher ordered state, which is irreversible. Formation of gels and aggregates are generally thought to be toxic, and are often associated with disease. We observed irreversibility of the Hfq foci in $\Delta gshAB$ bacteria at N-24. It is possible that we see Hfq foci forming gels or aggregates during N starvation in $\Delta gshAB$ *E. coli*. To confirm if this is caused by PTM of Hfq, we plan to purify Hfq and determine if it contains any PTMs. To do this, we will use a twin-strep tagged Hfq strain, allowing us to purify and compare Hfq in both WT and $\Delta gshAB$ at various timepoints during N starvation. This will allow us to confirm which PTMs (if any) occur at set time points through N starvation, and whether these are changed in the absence of glutathione.

6.4. How could glutathione influence N metabolism?

In this work, we showed that glutathione was upregulated during N stress (Figure 3.2). Interestingly, we did not observe any difference in growth rate or survival between $\Delta gshAB$ and WT *E. coli* during the first 24 hours of N starvation (Figure 3.5). Glutathione production is energetically intensive; production of 1 mol of glutathione requires 2 mol ATP¹⁸⁹. Although ATP is not necessarily limited during N starvation, it seemed unlikely that there was no biological significance to upregulation of glutathione during N starvation. When we

investigated further, we observed a decrease in viability in $\Delta gshAB$ *E. coli* following 48 hours of N starvation (Figure 3.10 A) and a large increase in recovery time upon N replenishment (Figure 3.9). This showed that glutathione was in fact required for long term survival during N starvation.

We observed that exogenous glutathione could be used by *E. coli* as an N source, with addition of exogenous glutathione supporting growth and delaying entry into N starvation (Figure 3.16). As exogenous glutathione could be used as a nutrient source under our conditions, we contemplated that this could also be true of endogenously produced glutathione. The current literature shows that bacteria recycle glutathione into cysteine for incorporation into the cytosolic amino acid pool, as well as using it as a sulphur source during nutrient deprivation¹³⁶. Interestingly, we observed that both cysteine and glycine concentrations (products of glutathione breakdown by GGT) were reduced in $\Delta gshAB$ compared to WT at N-24 (Figure 4.2). We also observed that addition of exogenous glutathione returned the early Hfq foci formation dynamics seen in $\Delta gshAB$ cells to near WT (Figure 3.15). We considered these results in the context of the wider literature, and considered the possibility that glutathione was upregulated during N starvation for utilisation later as an N source, and that this reserve N pool could affect Hfq foci formation. If $\Delta gshAB$ bacteria did not have a reserve N store, could they become N starved sooner than WT and this caused Hfq foci to form sooner?

WT and $\Delta gshAB$ *E. coli* displayed similar growth dynamics during the initial stages of N starvation and became growth attenuated at similar time points, suggesting that both strains entered N starvation at approximately the same time (Figure 3.5 A). This shows that $\Delta gshAB$ bacteria did not experience N starvation earlier than WT. To further investigate if glutathione's protective effect was due to its availability as an N source, we added an alternate poor N source (aspartate) to $\Delta gshAB$ cultures in place of glutathione (Figure 4.4); whilst this N source also supported growth and delayed entry into N starvation, it did not delay early foci formation in $\Delta gshAB$. Additionally, we generated mutant bacterial strains which could not catabolise glutathione (Δggt and $\Delta gshAB\Delta ggt$). Addition of exogenous glutathione still delayed early foci formation in $\Delta gshAB\Delta ggt$ bacteria (Figure 4.7), showing that break down of glutathione was not required to prevent early foci formation. We also wished to confirm if breakdown of glutathione was required for long term survival in WT and $\Delta gshAB$ *E. coli*. We observed no changes to the survival of either strain during long term N starvation when compared to their Δggt mutant counterparts. This showed that catabolism of glutathione was not necessary for long term survival of *E. coli* during N starvation.

Glutathione has been shown in the literature to protect many proteins against damage^{147, 167, 178}. Some of these proteins include those involved in N assimilation, sensing and response¹⁷⁶

¹⁴⁵. We hypothesised that these proteins may become damaged in the absence of glutathione during N starvation, and that this could dysregulate the cellular response to N starvation. This dysregulated response to stress could lead to the earlier foci formation and compromised survival we observed. This, of course, speculates that the Hfq foci have a practical role facilitating cell survival during N stress, a hypothesis which is currently under investigation. GlnG is a protein responsible for N sensing in *E. coli* (1.1.2) and interestingly, Hfq foci form sooner in GlnG knock out bacteria ⁸⁹. Therefore, GlnG would be an interesting protein to study when investigating changes to N metabolism. We also considered whether $\Delta gshAB$ bacteria had compromised ability to scavenge and assimilate N during longer term N starvation. Glutathione has been shown to protect against methylglyoxal (MG) toxicity, being required to break MG down into non-toxic metabolites. Interestingly, methylglyoxal can inactivate glutamine synthetase ⁵ by binding to arginine residues - this modification of GS is very stable. GS is an important part of the GS-GOGAT pathway, one of two major N assimilation pathways in bacteria. MG is known to inactivate GS, meaning it could potentially disrupt N assimilation *in vivo*.

LLPS proteins tend to disperse once cellular stress is alleviated, as it is no longer energetically favourable for them to exist in a phase separated state. The lack of dispersal upon addition of N to starved $\Delta gshAB$ also supports the hypothesis that the apparatus responsible for N assimilation could be damaged. $\Delta gshAB$ cells do not seem as responsive to N replenishment, the foci do not disperse, and recovery growth is severely compromised. We reason these dispersal and growth defects could be due to dysregulation in N sensing pathways, or the ability of the cell to enzymatically assimilate N. Future work would aim to test if $\Delta gshAB$ bacteria are still responsive to sensing changes in N. To determine this, AKG (which concentration decreases as N assimilation increases) and glutamine levels could be measured throughout N starvation and replenishment, to determine how effective N assimilation is in the mutant strain. Alternatively, Hfq foci may not be reversible due to damage of other components of the foci or cell components which influence their dispersal (this is further discussed in 6.5). Alternatively, the Hfq foci may have transitioned into a gel like state which is irreversible.

This data together suggested that whilst glutathione could be used as an N source by *E. coli*, the addition of glutathione likely prevented early foci formation by providing some other physiological protection or relief against another cellular stress. However, considering our data in the context of the wider literature, we speculate that glutathione could influence N metabolism in *E. coli* in some way, and this may affect long term survival and recovery of *E. coli* during N starvation.

Next, we will discuss the known roles of glutathione and the likelihood that they could be affecting Hfq foci formation and cell survival during N starvation.

6.5. The role of glutathione in the adaptive response to stress

6.5.1. Oxidative stress

As glutathione protects against oxidative stress, we considered whether the Hfq foci may be forming in response to ROS in WT bacteria, and that increased stress may lead to earlier foci formation in *ΔgshAB E. coli*. Unfortunately, contradictory to this hypothesis, the literature reports that Hfq foci are not induced by addition of ROS-inducers H₂O₂ or 2,20- dipyridyl⁵² at mid exponential phase. However, there are many factors affecting bacterial responses to oxidative stress. Susceptibility of glutathione mutants to H₂O₂ and superoxides appears to be dependent on growth stage and conditions^{153 128}: it has been demonstrated glutathione null mutants have normal resistance to H₂O₂ challenge during exponential phase¹⁴³ and only become susceptible to H₂O₂ during stationary phase¹³⁰. The existing data is conflicting and demonstrates how condition specific these responses are, therefore further work will have to be carried out before eliminating ROS as an influencing factor in early Hfq foci formation in *ΔgshAB E. coli*.

We began to investigate this possibility during this work, discovering that *ΔgshAB E. coli* were more susceptible to killing by H₂O₂ challenge during exponential growth (Appendix 8.1). To determine if Hfq foci formed early in *ΔgshAB E. coli* due to higher concentrations of ROS, we attempted to quantify the concentration of ROS in WT and *ΔgshAB* bacteria during N starvation. We faced challenges when attempting these experiments. Our lab previously observed that membranes of N starved bacteria are less permeable to small molecules, making the use of dyes to detect ROS difficult. Expression of reporter proteins from plasmids was also ineffective, as transcription and translation rates are low under these conditions, therefore induction during starvation was difficult. Bacteria also shifted towards catabolism and maintenance of resources during N starvation, often leading to degradation of the plasmid derived proteins. Detection of GSH:GSSG ratio is an alternative method used in eukaryotic cells to determine the redox state of the cells, as higher concentrations of ROS lead to an increase in oxidised glutathione (GSSG). This method is less useful in prokaryotes however, as current literature shows that GSH:GSSG ratios do not always change even when cells are under extensive oxidative stress^{128, 147}. To determine if *ΔgshAB* bacteria are under greater oxidative stress than WT, future work could involve RNA sequencing or reverse-transcription PCR to quantify changes in genes involved in the oxidative stress response.

We also challenged $\Delta gshAB$ bacteria with 10mM H_2O_2 at N-, and did not observe formation of foci following 1 hour – this result was supportive of the current literature. We therefore concluded that an increase of ROS does not cause Hfq foci to form earlier in these the $\Delta gshAB$ bacteria. Whilst it seems unlikely ROS were the trigger for formation of the Hfq foci directly, damage caused by oxidative stress may lead the foci to form more densely and earlier in the $\Delta gshAB$ strain. For example if ROS causes damage to RNA, DNA or proteins, this could induce LLPS (discussed in detail above). We discussed in 3.2.2.3.1 the damage caused by ROS to bacterial cells. Some proteins are more susceptible to damage by ROS, such as components of the N assimilation pathway; glutamate synthase and glutamine synthetase ¹⁴⁵.

We know from the literature that RNA is required for Hfq foci formation, and that changes in RNA type or the affinity of Hfq for RNA can change Hfq foci formation ⁵². We also know that PTMs caused by oxidative stress can affect LLPS of proteins (also discussed in detail above). Future work will attempt to compare RNA damage in $\Delta gshAB$ and WT *E. coli* during N starvation and determine if RNA damage correlates with Hfq foci formation.

6.5.2. Osmotic stress and low pH

It is known that glutathione plays a role in the response to both osmotic changes and low pH ^{161 129}. We therefore reasoned that the observed increase of glutathione during N starvation (3.2.1) may actually be a response to osmotic or pH changes. Osmotic pressure and pH are also known factors influencing the LLPS of proteins ⁴⁸. It is therefore not unreasonable to hypothesise that early Hfq foci formation in $\Delta gshAB$ *E. coli* was due to changes in pH or osmotic stress rather than a response specifically to N starvation. It is thought that the protective role of glutathione during osmotic stress may actually be a response to the oxidative stress which usually accompanies it. We therefore we cannot rule out that oxidative stress plays a role in Hfq foci formation, despite not seeing foci form upon addition of ROS ⁵². This is something we will investigate going forward.

Glutathione is known to prevent efflux of K^+ by binding to Kef channels (protein channels responsible for K^+ transport). In low K^+ media, glutathione null mutants show rapid loss of K^+ from the cell ^{169, 170}. Variation in K^+ concentrations alter cellular pH, which in turn can affect LLPS of some proteins. This occurs as the thermodynamics of the cell change when the pH of the cytoplasm nears the isoelectric point of a protein ¹⁹⁰. Proteins in other organisms have been shown to undergo accelerated LLPS during changes in pH, facilitated by pH sensing domains in the proteins ¹⁹¹. We therefore speculate that changes in pH caused by K^+ leakage could lead to altered LLPS of Hfq in the $\Delta gshAB$ mutant. However, our N starvation experiments were carried out in Gutnick minimal media (which contained high concentrations

of K⁺). This lessened the likelihood of extensive K⁺ leakage. Further experimentation is required to rule out K⁺ leakage from $\Delta gshAB$ as a driving factor for early Hfq foci formation.

6.6. What does this work tell us about the Hfq foci?

The literature shows that Hfq foci form in response to certain stresses, and there is a certain requirement for RNA binding for formation to occur ⁵². Interestingly, the length, structure, sequence and concentration of RNA can all effect properties of LLPS condensates ^{52 54, 55}. The secondary structure of mRNA determines whether it can self-associate, and be recruited or excluded from LLPS condensates ⁵⁶. We hypothesised that the Hfq foci may be acting as some form of metabolic break, to slow translation or protect specific mRNAs, which could be required for swift recovery once N is once again readily available. Future work to determine if the Hfq foci are enriched for particular RNAs would help provide insight in to their physiological function.

We observed the Hfq foci formed earlier in $\Delta gshAB$ bacteria (Figure 3.6) and they did not disperse upon addition of N (Figure 3.9). We also observed the mutant suffered a viability defect during prolonged starvation (Figure 3.10) and had an increased lag time when recovering from N stress (Figure 3.9 A). Due to the correlation between foci and growth and viability, we hypothesised that these changes in Hfq formation may be linked to survival. Previously we had been unable to test this hypothesis as Hfq is involved in a many cellular processes, therefore creating a Hfq null mutant creates too pleiotropic effects which may not relate to foci formation. Interestingly, we identified a $\Delta gshAB$ revertor mutant which no longer suffered reduced viability during long term N starvation. We characterised the Hfq foci formation and dispersal in the $\Delta gshAB$ revertor mutant and found that Hfq foci formation was unaffected compared to $\Delta gshAB$ (Figure 5.3), showing that foci formation did not correlate with the enhanced survival of this strain. However, we observed Hfq foci dispersal in the $\Delta gshAB$ revertor was no longer the same as that seen in $\Delta gshAB$, with foci dispersing by N+10 (Figure 5.4). From this data, we considered the hypothesis that the Hfq foci may need to disperse before *E. coli* could grow upon recovery from N starvation. In fact, we observed the opposite effect, with the $\Delta gshAB$ revertor showing an even longer lag time upon recovery from N starvation than both the WT and $\Delta gshAB$ (Figure 5.5), with the foci dispersing long before the cells recovered. This data suggests that the Hfq foci formation dynamics do not affect survival or growth recovery in our conditions, however this data is limited by the fact the revertor was not generated during a true suppressor screen. It is however a proof of concept, and a true suppressor screen will be carried out in the future.

We have observed that the Hfq foci depend upon TmaR for formation during N starvation, an observation also reported in Hfq foci which form during long term stationary phase, suggesting

that this phenomenon shares common formation properties in both stresses. TmaR is involved in glucose metabolism, and as glutathione has been implicated in protecting TCA cycle enzymes - this could implicate C (which would be present in excess during N starvation) in Hfq foci formation. It raises the question of whether the Hfq foci are forming in response to N starvation specifically, an excess of C, or damage to TCA and N assimilation enzymes. To demonstrate that the early Hfq foci formation in $\Delta gshAB$ is a direct consequence of N status in the cell, future work could include deleting the GAF domain of PtsP, making the cell blind to N status. There may be a common trigger where many of these stress responses converge, resulting in Hfq foci formation. These are all considerations which influence future work in this field.

We must continue to gain insights into the trigger and physiological way in which the Hfq foci form in order to determine their function.

6.7. Chapter summary

In our lab, we see the Hfq foci form in response to N starvation in *E. coli*. Others have reported the formation of Hfq foci in long term stationary phase and osmotic stress⁵². Interestingly, it has been shown that Hfq foci form in response to N starvation, however do not form in response to C starvation or direct oxidative challenge demonstrating that these structures are an adaptive response to some but not all stresses⁵². Whilst we often discuss adaptive stress responses as discrete processes, they are, in fact, not so clearly defined and many adaptive stress responses confer physiological resistance to a number of stressors – this is often termed cross protection. The adaptive responses to C starvation, low pH and oxidative stress have been shown to cross protect against each other¹⁹². It is therefore possible that the formation of Hfq foci is a component of a cross protecting response against stressors such as nitrogen starvation, stationary phase, cold shock and/or osmotic shock.

It is possible that the Hfq foci form canonically in $\Delta gshAB$ and the process is accelerated or exacerbated by exposure to an excess of another physiological stressor, such as oxidative, osmotic or low pH. This hypothesis is supported by the observation that $\Delta gshAB$ *E. coli* become growth attenuated at a similar timepoint as WT, following N runout (Figure 3.5 A), suggesting that they do not become N starved earlier than WT bacteria. This suggests that $\Delta gshAB$ bacteria are not more N starved than WT, therefore it is likely they are experiencing an excess of some other stress.

As there is currently little evidence demonstrating the function or full composition of the Hfq foci under any condition, it is possible that the composition and function of the Hfq foci are

different when they form under different stress conditions. Therefore, we are currently unable to make conclusive direct comparisons between Hfq foci in different stresses.

Ultimately, this work has demonstrated glutathione acts as a regulatory metabolite during N starvation, that glutathione affects both long term survival and Hfq foci formation during N starvation in *E. coli*, and that the precise mechanism by which this occurs is still unknown and subject to future investigations.

7. Chapter 7: Engineered chimeric natural products as novel antibacterials

7.1. Foreword

The following chapter was a separate project to the work shown in the rest of this thesis. We collaborated with Syngulon, an industry partner specialising in bacteriocin biology, to utilise our knowledge of viral small proteins and their knowledge of bacteriocins to attempt to create a novel alternative to antibiotics. The initial results, although of use and interest, did not provide a clear path towards publication, translation, or PhD thesis. Due to travel restrictions imposed during to COVID, we were unable to capitalise on Syngulon's expertise in developing the experiments further. Hence, we decided to discontinue the experiments 18 months into my PhD programme and embark on the research described in chapters 3, 4 and 5.

7.2. Introduction

7.2.1. The problem of antimicrobial resistance

Antimicrobial resistance is one of the most pressing health issues faced by society ¹⁹³. Traditional antibiotics have been overused in both medicine and agriculture, leading to the emergence of bacterial strains which are resistant multiple antibiotics. It has been estimated that approximately 1.27 million people worldwide died directly due to AMR in 2019 ¹⁹³, with the WHO predicting this will rise to 10 million by 2050. As well as the human cost, there is also a huge economic cost to treating AMR infections.

The misuse of antibiotics in human medicine, as well as animal and plants has led to the development of resistant pathogens. Many conventional treatments are becoming ineffective, not only due to the rise in resistance mechanisms but also practical issues such as limited infection site penetration ¹⁹⁴. Many traditional antibiotics target similar cellular mechanisms, meaning bacteria can easily evolve strategies to avoid cell death. Novel antibiotics are difficult and expensive to produce, and alternate strategies are required to meet current need. A report commissioned by the Wellcome Trust in 2016 highlighted the requirement for phage inspired and antimicrobial peptide solutions to this global problem ¹⁹⁵. Research over the last decade has identified countless naturally occurring proteins with antimicrobial properties ^{127, 196}. However, the nature of these proteins and structure of bacterial cell walls means they are still vastly underutilised as therapeutics, as there remains a difficulty in targeting these antimicrobials to bacterial cells. Whilst new delivery systems, such as nanoparticles laden with antibacterials, have been developed they do not appear to have increased the efficacy of therapeutics, particularly when attempting to deliver cargo across the complex gram-negative bacterial cell inner membrane ¹⁹⁴. Due to the ongoing emergence of antibiotic resistant bacteria, the need for novel antimicrobials remains a pressing issue.

7.2.2. Naturally occurring antimicrobials

7.2.2.1. Viral antimicrobials: T7 phage host acquisition factors

Obligate bacterial intracellular viruses (bacteriophage) are abundant in nature ¹⁹⁷. Bacteriophages depend upon their host for reproduction and metabolic processes, and their lifecycles vary greatly between species. However, most follow the same basic stages during viral infection and replication (Figure 7.1 A). These phages use bacterial machinery to express small proteins from their own genome to repurpose bacterial processes, ensuring infection and production of progeny ¹⁹⁸⁻²⁰⁰. These proteins, known as host acquisition factors (HAFs), are abundant in nature and play roles in modulating host transcription, motility, metabolism, protein homeostasis, RNA degradation, cell division and CRISPR systems ²⁰⁰⁻²⁰².

One such well-studied bacteriophage is the obligate *E. coli* phage T7. T7 phage is a lytic double stranded DNA virus, with a genome of approximately 40 kb encoding 56 genes ²⁰³. The T7 phage lifecycle can be broadly separated into three phases: attachment, transfer of DNA and gene expression, lysis and release of phage progeny. During these phases, T7 phage expresses HAFs in a highly regulated order (Figure 7.1 B). These HAFs are classified as early (Class I), middle (Class II) and late (Class III) genes, depending on their order of expression during infection.

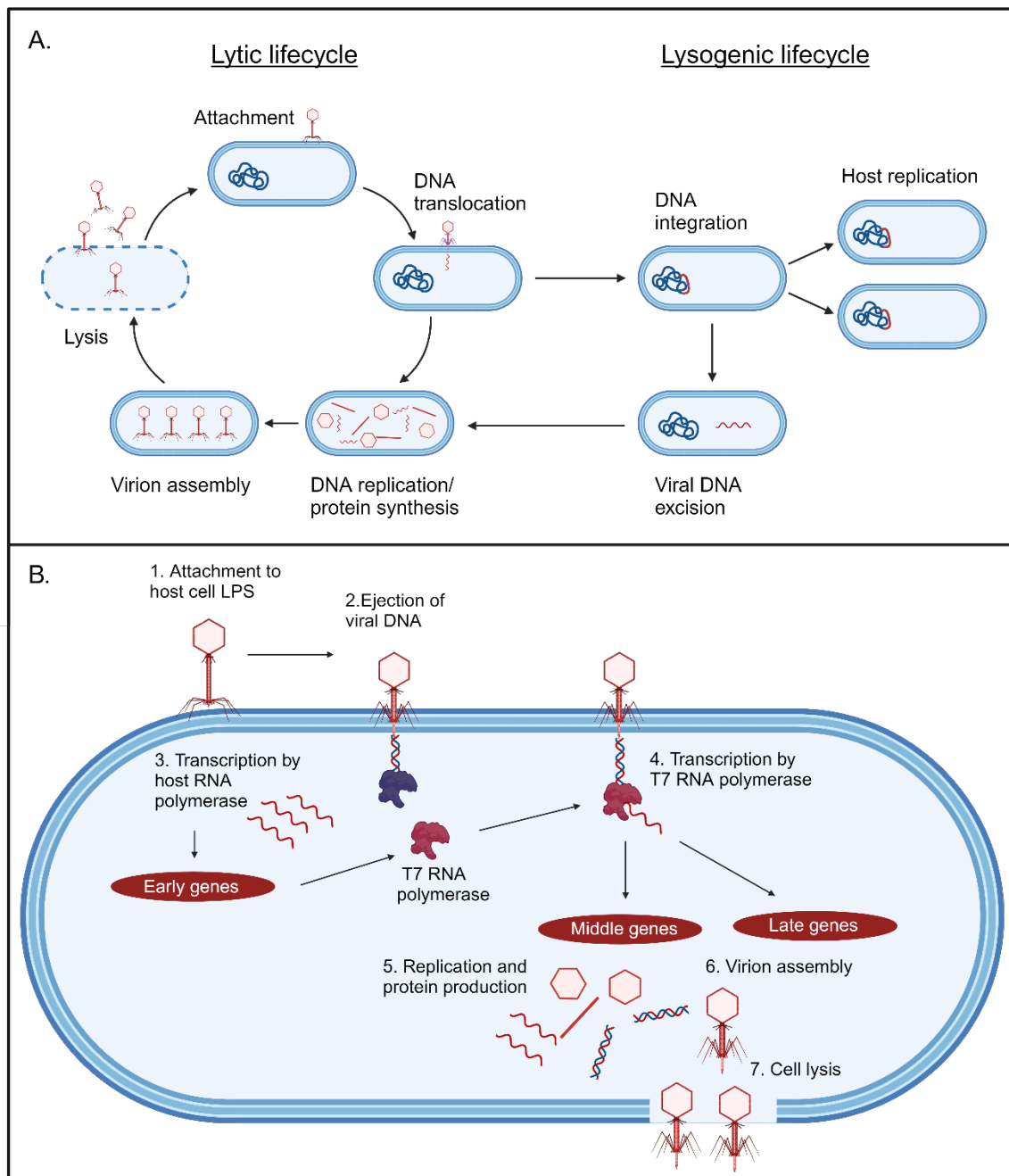


Figure 7.1: Bacteriophage lifecycles

(A) Most viruses follow the lytic or lysogenic lifecycles. During the lytic cycle, the virus enters the cell where it hijacks host replication machinery. Viral genetic material is replicated and packaged into self-assembling protein coats. The infected cell continues to produce virions until it eventually lyses, releasing the newly made virus. During the lysogenic cycle, viral genetic information is inserted into the host genome. Viral gene expression is prevented by repressor genes located in the viral genome. When the host cell replicates, the viral genetic information is replicated alongside its own. Eventually, the host cells recognise the foreign viral DNA and host cell machinery excises the foreign nucleic acid, triggering the lytic cycle.¹⁰ (B) T7 phage lytic replication lifecycle. Viral DNA is initially transcribed by host RNAP before it is inhibited by viral early proteins. Transcription of middle and late genes is carried out by T7 RNAP. Replication of viral DNA and protein production leads to formation of new T7 virions. Late genes produce proteins responsible for cell lysis.

During the first phase, T7 phage initially attaches by binding to a receptor on the host cell, the host lipopolysaccharide, via its C terminal tail fibre protein (Gp17) ²⁰⁴.

During the second phase, phage DNA is transferred into the cell. Translocation of T7 DNA into *E. coli* is a transcription coupled process, requiring the housekeeping sigma factor (RpoD, also referred to as σ^{70}) bound RNAP ⁷ to transcribe genes from the T7 early promoters ²⁰⁴. Approximately 850 bp of viral DNA is injected into the host cells, with the remaining genome being internalised during transcription by the host and then T7 RNAP ^{204, 205}. After initial infection, T7 utilises the host transcriptional machinery to express its own genes ²⁰⁶. The first gene to be transcribed by the host machinery is Gp1, which encodes the T7 RNA polymerase. T7 RNAP initiates transcription from 17 other promoters. Middle genes are all transcribed by T7 RNAP, these genes encode essential T7 replication proteins ²⁰⁷.

It is important for T7 phage to shut down the host RNAP, to prevent competition with its own viral RNAP when expressing middle and late genes. T7 phage achieve this through expression of middle gene proteins; Gp0.7, Gp2 and Gp5.7 ²⁰⁸. These HAFs are essential for the infection of *E. coli* and the production of viral progeny ^{201, 208}. In *E. coli*, the RNAP core enzyme consists of two α subunits (α and α'), two β subunits (β and β') and ω subunit ²⁰⁹. These subunits form what is referred to as the core enzyme. Promotor specificity is conferred by the binding of one of seven sigma factors, such as σ^{70} which is responsible for transcription of housekeeping genes, to form the holoenzyme. This active form of RNAP recognises promoters on DNA. Once bound to DNA, RNAP undergoes a conformational change from the closed to the open complex, and transcription can commence ⁷. Gp2 inhibits RNAP by binding to the β' subunit of the *E. coli* RNA polymerase, a negatively charged strip of amino acids facing the downstream binding channel. This causes steric and electrostatic inhibition of RNP polymerase, preventing transition from the RNAP closed complex (RPC) to the open complex ⁴, therefore halting transcription (Figure 7.2) ^{4, 201}. Together, the early gene Gp0.7 and middle gene Gp2 fully shut off activity of the *E. coli* $E\sigma^{70}$ and $E\sigma^{38}$ (the main stress response sigma factor) ⁷, leaving the T7 RNAP to transcribe subsequent genes and produce the proteins necessary to build and assemble new virions ^{201, 208}.

During the third phase, T7 late genes are involved in lysis of the host cell. These proteins include holin (Gp17.5) and lysozyme (Gp3.5). Holin is a small membrane protein which accumulates within the host inner membrane, eventually causing holes, which is thought to control the timing of lysis ²¹⁰. The timing of lysis is very important for a phage lifecycle, as a short lysis time can lead to less viral progeny being released.

As discussed, T7 phage HAFs act against a multitude of processes within the host cell. Gp2 is a well studied, small (7 kDa), negatively charged viral protein able to effectively inhibit

transcription in *E. coli*, thereby halting bacterial growth²⁰¹ - thus, Gp2 is a promising candidate for use as an antimicrobial agent.

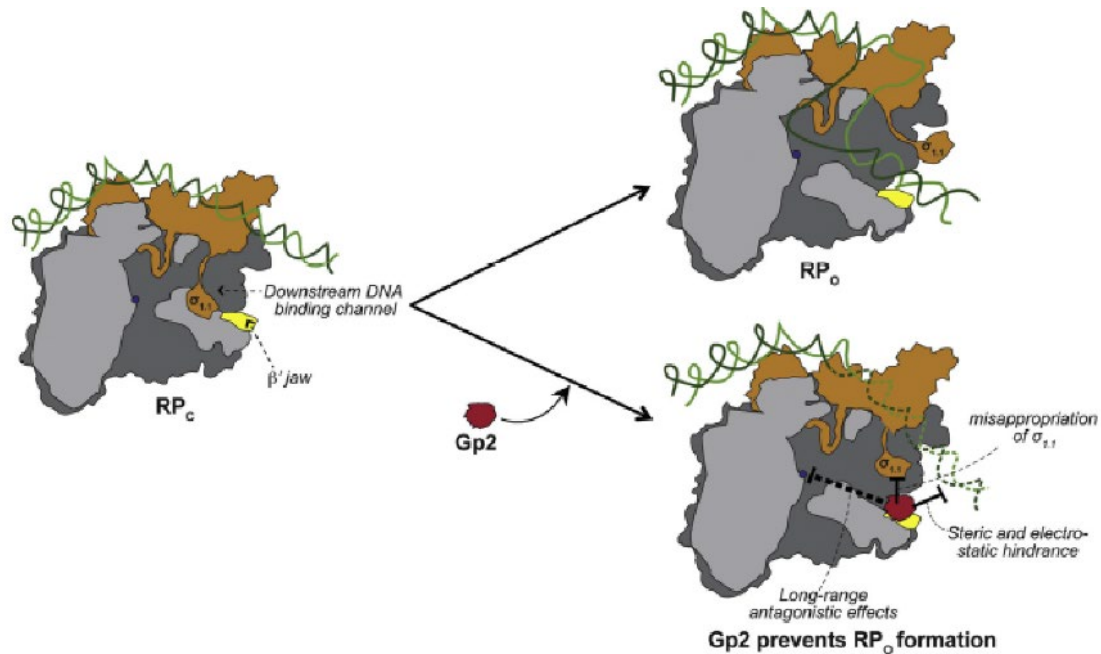


Figure 7.2: Inhibition of *E. coli* host RNAP by T7 phage in exponentially growing cells

(A) Schematic showing how T7 phage inhibits *E. coli* RNAP during exponential growth. (B) Schematic summarising the binding of Gp2 to $E\sigma_{70}$, preventing open complex formation. Figure from Tabib-Salazar et al.⁴

7.2.3. Bacterial antimicrobials: bacteriocins

There are a wide variety of drug delivery mechanisms currently utilised in both medicine and food production, ranging from the delivery of lysins to *P. aeruginosa* by the colicin pyocin S2²¹¹, to small delivery nanoparticles and cell penetrating peptides²¹². For further detail, novel delivery mechanisms have been extensively reviewed in the literature^{194, 213, 214}.

Bacteriocins are small antimicrobial peptides, approximately 20-60 amino acids in length, produced by both gram positive and gram negative bacteria to inhibit sensitive strains of competing bacteria, providing a competitive advantage in their natural environments²¹⁵. Genes encoding bacteriocins occur in operons within the bacterial genome or on mobile genetic elements such as plasmids²¹⁶. These operons also contain bacteriocin immunity

proteins, which protect the producing bacteria ^{216, 217}. Expression of bacteriocin genes is highly regulated, usually by multi-component regulatory systems ²¹⁸.

Bacteriocins are divided into three classes based on their antibacterial activity, transport mechanism and receptor interactions ¹⁹⁶. Class I bacteriocins, such as Nisin, are typically 19-50 amino acids and are extensively post-translationally modified ²¹⁹. Class II bacteriocins are smaller, unmodified proteins which are generally more heat stable ²¹⁵. Class III bacteriocins are large peptides (approximately 15 kDa or above) which typically have complex domain structures, with each domain conveying a different function/property ²¹⁵.

Bacteriocin mechanism of action can vary from membrane disruption to nuclease activity within the cytoplasm ^{211, 215}. Here we focussed on the latter, those with nuclease activity - specifically Colicins.

7.2.3.1. Colicins: production, regulation and mechanism of action

Colicins are a type of Class III bacteriocin produced by strains of *Escherichia coli* containing the pCol plasmid ^{196, 220}. Colicins are active against *E. coli* and other closely related bacteria such as *Shigella boydii* (colicin U) and *Citrobacter freundii* (colicin A) ^{196, 220}. Generally, they are produced in high volumes and secreted into the extracellular space ²²⁰. As with other bacteriocins, colicins are produced with an immunity protein, which renders the protein non-toxic to the producing cell. These immunity proteins have separate promoters, allowing production at much higher concentrations than the colicin, resulting in little to no free colicin within the cell ¹⁹⁶.

There are two types of colicins, Group A and Group B ¹⁹⁶; the structure and functions of these colicins are very diverse (Figure 7.3 B-F). Group A colicins are secreted proteins encoded by small plasmids (approximately 10kb) with a relatively high copy number and enter cells via the Tol system ¹⁹⁶. Group B colicins are encoded by large plasmids (approximately 40kb) usually with just one copy per cell, these plasmids can contain more than one colicin operon. Unlike Group A colicins, Group B colicins are not secreted ¹⁹⁶.

In this work, we focussed on Group A secreted colicins, specifically E colicins, as they have several properties which could potentially be exploited for antimicrobial peptide delivery. We will discuss these properties below.

There are two types of E colicins, those with pore forming activity and those with enzymatic nuclease activity. Both pore forming and enzymatic nuclease E colicins are regulated by the SOS promoter ¹⁹⁶. Under normal conditions, the operon is regulated by the LexA repressor ¹⁹⁶, when cells undergo stress, the SOS response is initiated and production of colicin begins ¹⁹⁶. Colicin concentration can increase by 1000-fold in around 90 minutes ²²¹.

The colicin immunity protein protects the producing cell by binding to the colicin receptor domain before secretion and remains in complex until the colicin undergoes a conformational change, caused by binding to the appropriate receptor on a target bacterial cell ^{196, 222}. Once the immunity protein is released, the colicin is transported into the target cell ^{220, 222}. Whilst the immunity gene, *cxi* is found downstream on the colicin operon, it is under the control of a separate promoter allowing it to be produced at much higher concentrations than the colicin, resulting in no free colicin within the producing cell ¹⁹⁶.

The action of colicins are largely determined by their domain structure. E colicins can pass through the outer membrane, where they either form pores in the inner membrane causing cell lysis, or pass through the inner membrane to the cytoplasm ¹⁹⁶. All E colicins follow a specific domain structure, shown in Figure 7.3 (A). The receptor (R) domain facilitates high affinity binding to receptors on target cell surfaces (found on *E. coli* and closely related bacteria), the transport (T) domain facilitates transport of both T and cytotoxic (C) domains through the outer membrane via complexes of transport channel proteins. The cytotoxic domain, responsible for cytotoxicity, enters the cytoplasm through the inner membrane where it has its effect ^{196, 222}. Colicin E2 and E3 utilise the BtuB/Tol machinery to enter the bacterial cell. Once the cytotoxic domain has traversed the membrane, it is cleaved at residue 420 by the inner membrane protein FtsH (Figure 7.3 G) ²²³.

The cytotoxic domain of enzymatic colicins have nuclease activity in the cytoplasm and with activity varying depending on the colicin - for example colicin E3 has specific RNase activity against 16S rRNA and colicin E2 has DNase activity ^{125, 196}.

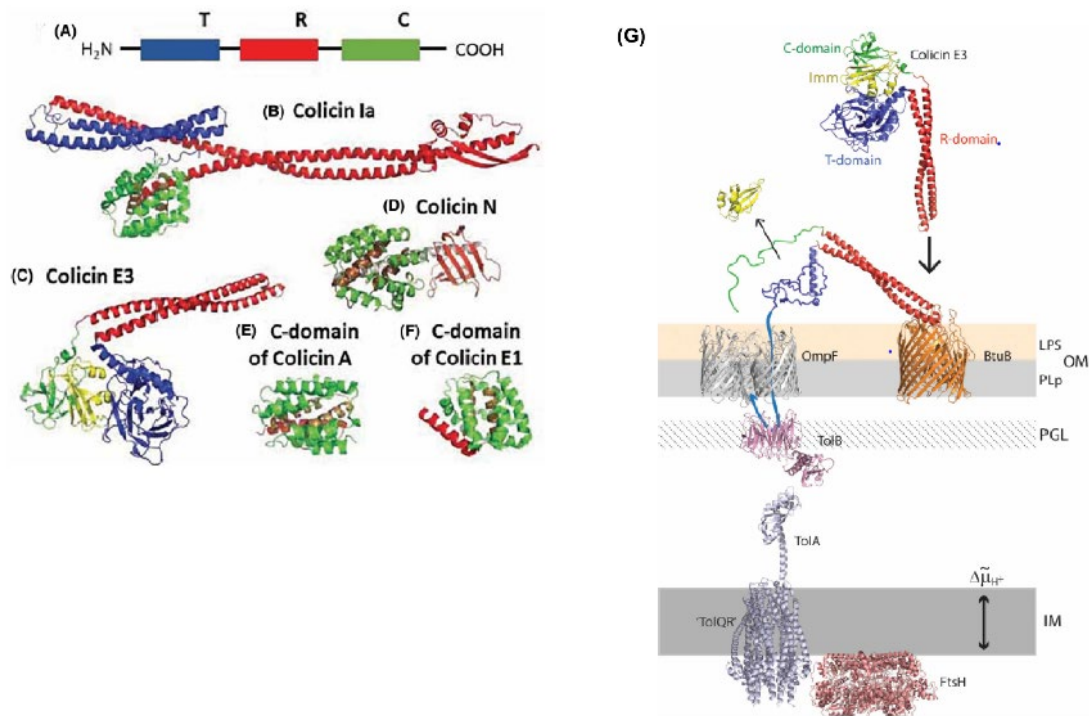


Figure 7.3: Structure of colicins

(A) Functional domains of Colicin E2 with T (transport domain), R and C terminal cytotoxic domain shown. (B-F) Ribbon schematics showing the variety of structure of colicins (B) Ia, (C) colicin 3, (D) colicin N, (E) colicin A C-domain, (F) colicin E1 C-domain. Yellow ribbon depicts the immunity protein bound to enzymatic colicin C. (G) Colicin E2 entry mechanism. Figure adapted from Cramer et al. ^{6 9}

7.2.3.1.1. Colicin E2 as a drug delivery vehicle

Colicin E2 is an enzymatic colicin consisting of the three-domain structure previously described (Figure 7.3 A). After secretion from the producing cell, the receptor domain binds with high affinity to the BtuB receptor on the target cell. This complex recruits OmpF proteins, which facilitate transport of the T-domain into the periplasmic space. The transport domain interacts with the TolA and TolB proteins in the periplasmic space (Figure 7.3 G). It is suggested that the T domain facilitates transport of the cytotoxic domain to the inner membrane, however it is unknown how the C-domain passes through the inner membrane. Although much remains unknown about how the C-domain traverses the inner membrane, it has been shown that once in the cytoplasm the colicin E2 cytotoxic domain is cleaved by the ATP-dependent zinc metalloprotease, FtsH (Figure 7.3 G), at around residue 420 (approximately 30 amino acids upstream of the cytotoxic domain) ^{222, 223}. Residues 452 and

456, at the border of the receptor and cytotoxic domain, have also been shown to impact successful cleavage ²²².

Whilst Colicins already have antibacterial properties allowing them to deliver their own cytotoxic cargo in to target cells, they cannot be effectively utilised in food production and medicine due to widespread occurrence of the gene and its corresponding immunity protein in *Escherichia coli* ¹⁹⁶. By replacing the cytotoxic domain of the protein with a range of small cargo proteins such as Gp2, we aimed to overcome this immunity and create a chimeric protein delivery system which would enable us to utilise a range of previously underutilised naturally occurring antibacterial proteins. As previously stated, the mechanism of transport of C-domain through the inner membrane is unestablished. However, studies have suggested isoelectric charge ²²⁴ and interaction with lipopolysaccharides of the inner membrane may play a role in binding and unfolding of the enzymatic C domain ²²⁵.

There are many naturally occurring small viral proteins, such as Gp2, which have an antibacterial effect. If a suitable delivery system could be developed this would allow us to exploit a vast supply of otherwise unusable antibacterial proteins. Further proteins would be identified as potential cargo candidates once information had been gathered on structural qualities making small proteins suitable for transport by colicin E2. Small viral proteins such as Gp2 are unable to pass through the bacterial membrane unaided, however once inside the cell these HAFs are able to target a wide range of bacterial cell machinery and often have very strong activity - therefore these HAFs provide ideal cargo candidates.

7.3. Chapter Aim

We proposed to utilise naturally occurring bacteriocins to create a delivery system for small proteins into gram-negative bacteria. The ability of bacteriocins to penetrate both membranes of gram-negative pathogens has not yet been exploited for production of novel antibacterials. This project aimed to deliver these small antimicrobial proteins using colicin E2 as a delivery vehicle. We proposed to replace the cytotoxic domain of colicin E2 with HAFs such as Gp2 (Figure 7.4 B). Our hypothesis was that the receptor and transport domains would remain unaffected by this C-domain substitution and would transport the antibacterial peptide into *E. coli*, where it would be cleaved by FtsH and inhibit RNA polymerase. Our initial approach engineered Colicin E2, a multi-domain bacteriocin which delivered its cytotoxic domain into the cytoplasm of gram-negative bacteria, replacing the cytotoxic domain with an alternative antibacterial cargo protein (experimental strategy shown in Figure 7.4). Colicin E2 has been shown to be effective against pathogenic strains of *E. coli*, including EHEC O157:H7²²⁶. Initially, we used an extensively studied T7 bacteriophage protein Gp2 as cargo, a 7 kDa early-stage protein which inhibits RNA polymerase^{4, 198}.

This work was carried out in collaboration with Syngulon, a Belgian company specialising in bacteriocin-based technologies. Syngulon have extensive experience working with colicins and were able to provide us with genetic sequences we required and subject expertise.

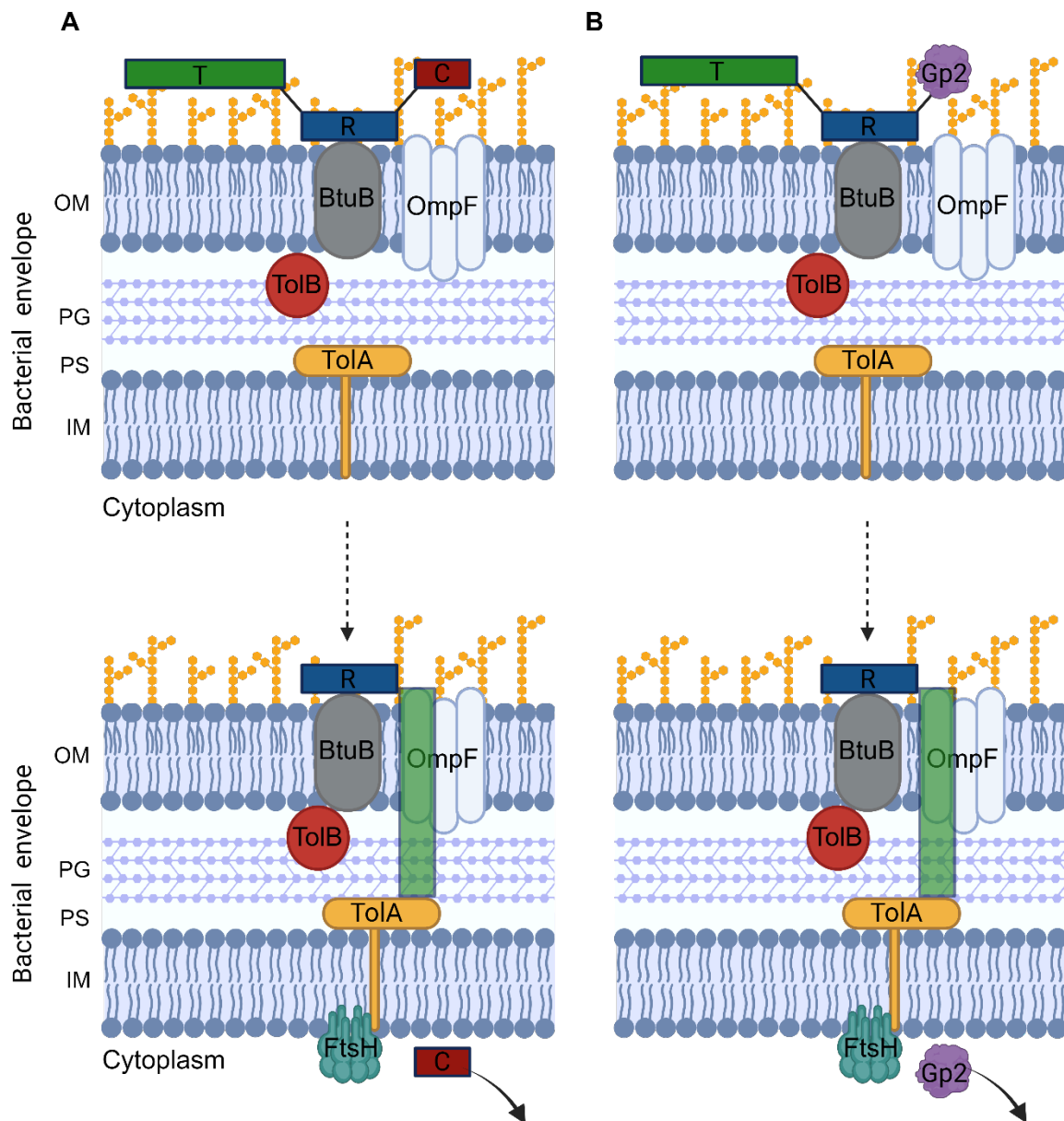


Figure 7.4: Graphical representation of colicin E2 and chimeric protein entry to cell

(A) Graphical representation showing mechanism of entry of colicin E2 in to target cell (B) and proposed replacement of cytotoxic domain with alternative antimicrobials such as Gp2.

7.4. Engineering chimeric natural products as novel antibacterials

7.4.1. Design of chimeric proteins

To investigate whether we could successfully use colicin E2 as a delivery mechanism of small proteins to bacterial cells, we initially designed one chimeric protein. Residues 420-456 have been shown to be necessary for colicin entry into the cell, we therefore placed Gp2 after residue 456 with a small amino acid linker. We also introduced a C-terminal his tag to Gp2. Previously, our lab had attempted to tag Gp2 at the N terminus, however Gp2 did not show measurable activity when tagged at the N terminus with other proteins (data not shown). We therefore introduced a His tag to the C terminus of Gp2 to provide a system for both protein purification and protein identification in future experiments.

As seen in Figure 7.5, Gp2 was placed downstream of residue 420 of the receptor domain. Residue 420 was previously described as the cleavage site for colicin E2²²². The intent of this structure was to allow Gp2 to be cleaved once inside the target cell, allowing it to carry out its inhibitory activity.

The pUC57-Colicin-E2 plasmid was provided by Syngulon; Gibson cloning was used to insert C-terminally His-tagged Gp2 or an inactive Gp2 mutant (R56E) downstream of an arabinose inducible promoter. Colicin E2-Gp2 and Colicin E2-R56E constructs were both cloned into the pUC57 vector (used for protein purification) and pBAD18 vector. All sequences were confirmed using Sanger sequencing.

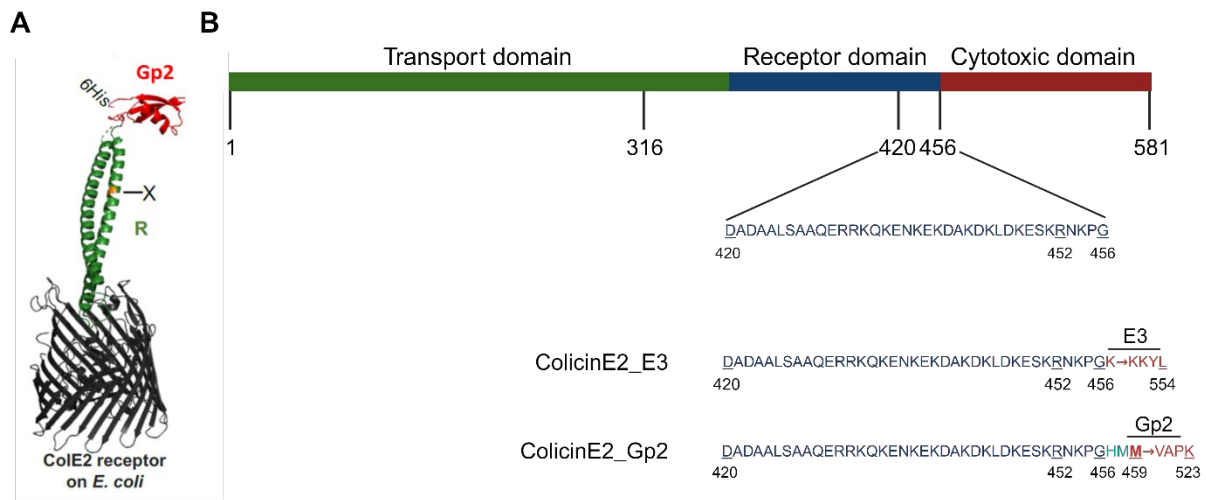


Figure 7.5: Structure of wild type colicin E2, E2-E3 and Colicin E2-Gp2 structures

(A) Structural model of colicin E2 bound to its receptor on *E. coli*, replacing the cytotoxic domain of colicin E2 with Gp2. (B) Schematic displays the three domains of colicin E2 (transport, receptor and cytotoxic). Residues important for efficient cleavage (420, 452 and 456) are highlighted. Colicin E2-E3 and ColicinE2-Gp2 show the design of each chimeric protein.

7.4.2. Characterising Colicin E2-Gp2

7.4.2.1. Colicin E2-Gp2 inhibits exponentially growing *E. coli* when expressed *in vivo*

To determine the ability of the C-terminally His tagged Colicin E2-Gp2 to attenuate bacterial growth, we cloned our chimeric protein construct downstream of an arabinose inducible promoter in the pBAD18 vector system ²²⁷. We performed growth inhibition assays using a multi-well platform in two strains of *E. coli*. MC1061 *E. coli* (which transports arabinose but does not metabolise it ²²⁷) and a standard *E. coli* lab strain MG1655. For induction from the araBAD promoter, we treated cultures with 0.2% v/v arabinose ²²⁷.

We induced our chimeric constructs and unaltered Gp2 in growing *E. coli*. The induction of Colicin E2-Gp2 in exponentially growing *E. coli* resulted in fast attenuation of growth of MC16061 (Figure 7.6 A). This growth attenuation was similar to that caused by the wild type, untagged Gp2. Importantly, Colicin E2-R56E mutants did not inhibit growth in this strain, demonstrating that this inhibition is driven by the active Gp2 in the chimeric protein (Figure 7.6 A). These results also demonstrate that adding a His tag to the C terminus of Gp2 does not inactivate Gp2 when expressed *in vivo*.

For future experiments, we wanted to use a standard lab strain of *E. coli* (MG1655). We therefore repeated this experiment in MG1655 bacteria to ensure that our chimeric protein behaved the same way in this strain. The induction of Colicin E2-Gp2 in exponentially growing MG1655 *E. coli* also resulted in fast growth attenuation (Figure 7.6 B). Colicin E2-Gp2 and wild type Gp2 caused similar growth attenuation, whilst the Colicin E2-R56E protein did not cause growth attenuation. For further work, we used *E. coli* MG1655 only.

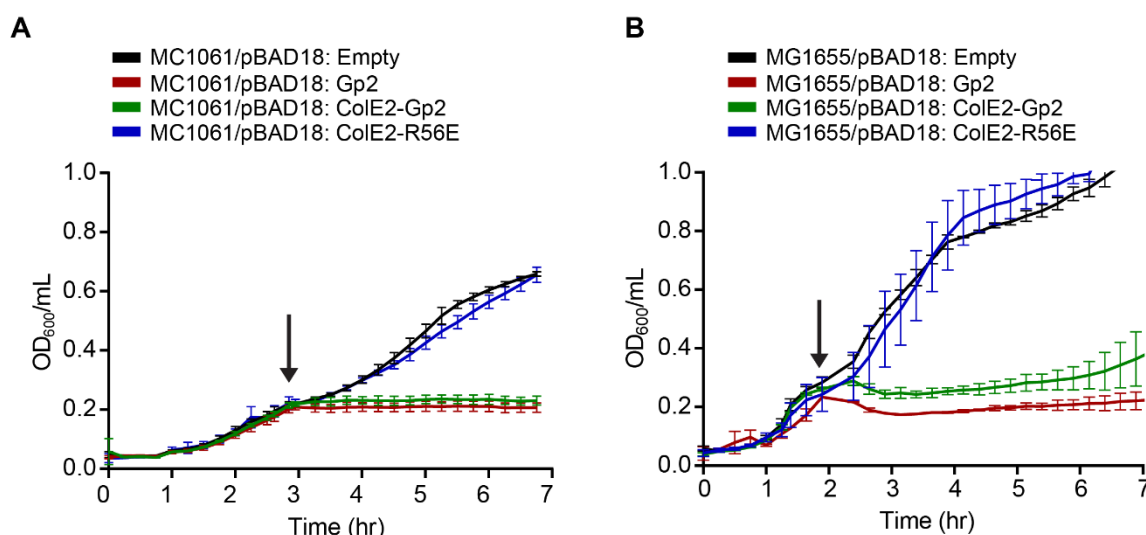


Figure 7.6: Colicin E2-Gp2/R56E attenuates growth of MC1061 and MG1655 *E. coli* when expressed *in vivo*

Growth measured by OD600nm of *E. coli* in LB. Gp2, Colicin E2-Gp2 and Colicin E2-R56E were expressed from pBAD18 plasmids, arrows indicate induction of plasmids by addition of 0.2% L-arabinose at OD600 0.2. (A) MC1061 and (B) MG1655. Error bars represent standard deviation (n=3).

7.4.2.2. Purification of Colicin E2-Gp2

After establishing intracellular Colicin E2-Gp2 was able to attenuate the growth of *E. coli*, we wanted to test the ability of Colicin E2-Gp2 to deliver its cargo Gp2 to target bacteria. To do this, we needed to treat bacterial cultures with exogenous chimeric proteins.

To ensure a high yield of protein, we purified Colicin E2-Gp2 using a His-Trap column, able to bind to the C-terminal His tag introduced to Gp2. Colicin E2-Gp2 was expressed in JE1(DE3) *E. coli* from a T7 promotor on the pUC57 plasmid. This strain contains an IPTG inducible T7 RNA polymerase able to recognise the promotor site on the puC57 expression plasmid. After

expressing our protein of interest for 4-5 hours, cells were harvested and lysed. After separation of soluble and insoluble lysate, soluble lysate was loaded on to an AKTAPure.

Samples of culture before and after induction, insoluble and soluble lysate and purified protein fractions were run alongside previously purified Gp2 (Figure 7.7 A). We confirmed the purified protein was the appropriate size as per our chimeric design (approximately 55 kDa). We further confirmed purification of the correct protein by western blot with an anti-Gp2 antibody (Figure 7.7 B).

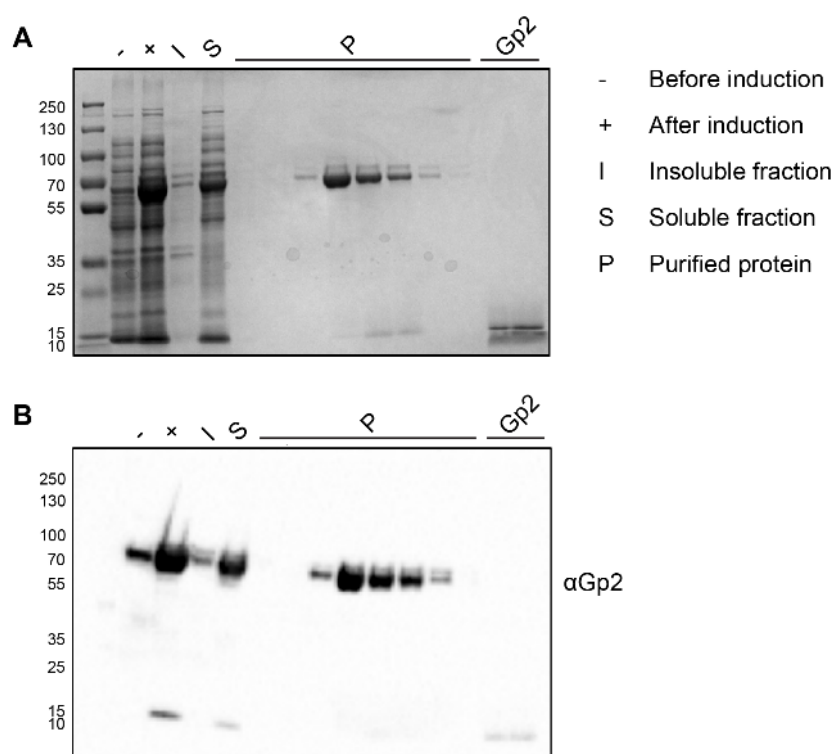


Figure 7.7: Representative Coomassie gel and western blot of protein purification

Representative (A) Coomassie stain and (B) immunoblot of extracts of *E. coli* expressing Colicin E2-Gp2: cells sampled before (-) and after ³ protein induction with 1mM IPTG, insoluble (I) and soluble (S) lysate fractions and purified protein fractions (P). Extracts and purified protein were run alongside purified Gp2 and stained with (A) Coomassie blue or (B) probed with anti-Gp2 antibody.

7.4.2.3. Purified Colicin E2-Gp2 protein inhibits bacterial RNA polymerase

It is common that many proteins misfold and lose their activity during the purification process. After successful purification of our protein, we wished to confirm whether the activity of Gp2 was retained.

Our lab has previously utilised an *in vitro* transcription initiation assay to determine the activity of purified RNAP inhibiting HAFs ^{201, 208}. This assay measures the activity of RNA polymerase, by determining the amount of radiolabelled transcript produced from the T7 early gene A1 promotor. All DNA probes using in this transcription initiation assay were 65 bp long, 20bp of which were downstream of the +1 transcription start site. To determine if our purified Colicin E2-Gp2 proteins affected the catalytic activity of *E. coli* RNAP, we measured the activity of $E\sigma^{70}$ in the presence of each purified protein using WT Gp2 as a positive control.

High levels of CpApU* transcripts were observed in the no Gp2 negative control, while a reduction in transcription was observed in the 1:2 RNAP:Gp2 positive control (Figure 7.8 left). When Colicin E2-Gp2 was pre-incubated with $E\sigma^{70}$ prior to addition of the DNA probe, we observe a substantial decrease in the ability of RNAP to produce transcripts. Inhibition occurred in a dose dependent manner, starting at a ratio of 1:1. Purified Colicin E2-R56E (Figure 7.8 right) did not inhibit the ability of RNAP to produce transcripts, demonstrating that this inhibition is driven by the active Gp2 in the chimeric protein.

We therefore conclude the Gp2 activity of the purified chimeric protein is retained, and the growth inhibition of *E. coli* seen in Figure 7.6 was likely caused by the inhibitory activity of Gp2 on RNA polymerase. These data together strongly suggest post-translational folding of Gp2 is not affected when it is part of the Colicin E2 chimeric protein.

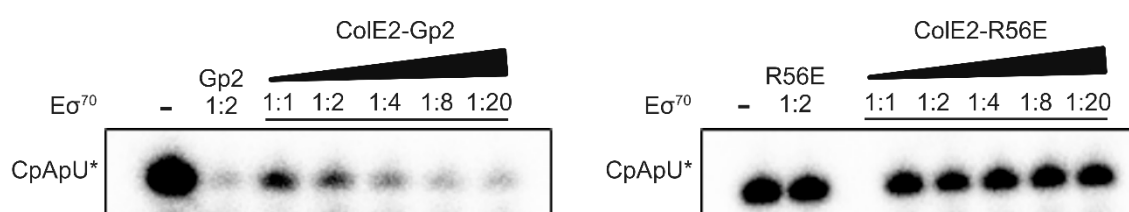


Figure 7.8: The effect of Colicin E2-Gp2 on *E. coli* RNA polymerase activity

Representative autoradiographs of denaturing urea gels, showing the effect of purified ColicinE2-Gp2 and ColicinE2-R56E to inhibit the ability of the *E. coli* RNAP holoenzyme to synthesis dinucleotide primed RNA product from the T7 A1 promotor ⁷. Radiolabelled nucleotides used in this assay are indicated by asterisk. RNAP concentration 400 nM per reaction.

7.4.2.4. Purified Colicin E2-Gp2 does not inhibit exponentially growing *E. coli*

The aim of this project was to deliver HAFs to bacteria using a chimeric colicin protein as a delivery mechanism. Once we had confirmed we could express and purify our chimeric Colicin E2-Gp2, and that Gp2 retains its activity, we wanted to determine if addition of exogenous Colicin E2-Gp2 would inhibit growth of *E. coli*. To do this, we treated cultures of *E. coli* with purified Colicin E2-Gp2 during exponential phase. Initially, cultures were treated with a range of protein from 0.02 μM to 1.4 μM . Growth inhibition was not observed upon addition of Colicin E2-Gp2 (Figure 7.9 A). To ensure this result was not due low protein concentrations, the experiment was repeated using a higher concentration of Colicin E2-Gp2; however, we still did not observe any growth inhibition (Figure 7.9 B), even at 4 μM .

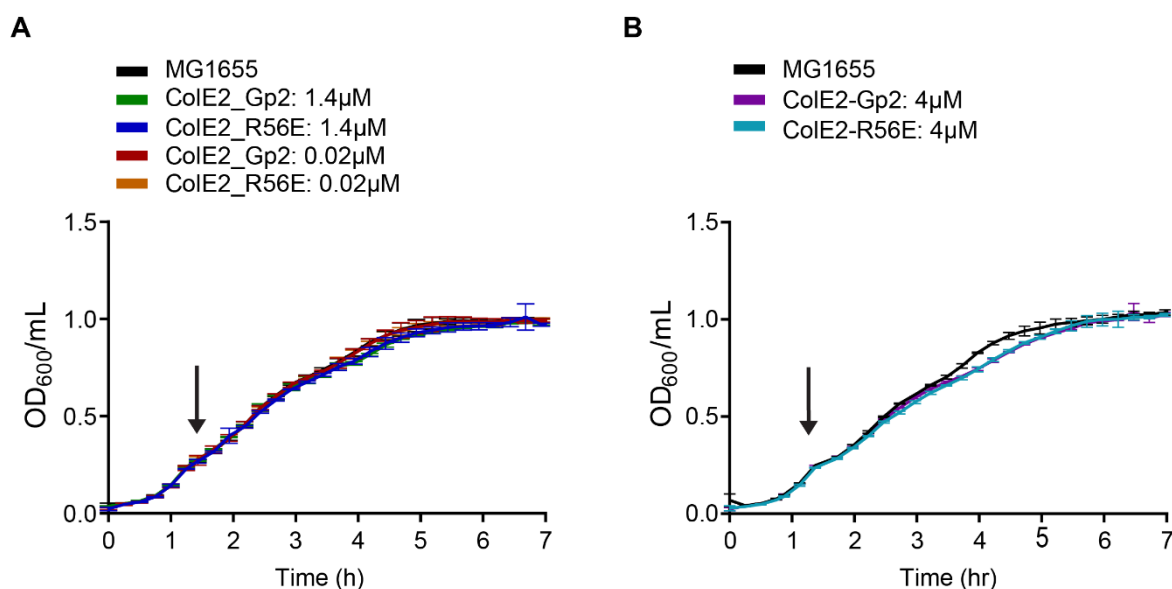


Figure 7.9: Exogenous Colicin E2-Gp2 does not inhibit exponentially growing *E. coli*

(A-B) Growth measured by OD₆₀₀nm of *E. coli* in LB. Arrows indicate where cultures were treated with purified Gp2, Colicin E2-Gp2 and Colicin E2-R56E. Error bars represent standard deviation (n=3).

As our original chimeric design of Colicin E2-Gp2 did not inhibit *E. coli* growth, we began to reconsider our experimental approach.

7.4.2.5. Cell free protein production

Previous experiments lacked a positive control, as colicins are extremely toxic and difficult to purify. Without such control, we were unable to confirm where or how our Colicin E2-Gp2 protein delivery system had failed.

Our collaborators at Syngulon had begun to use a new experimental approach, producing colicins using *in vitro* transcription and translation. Syngulon had successfully expressed a hybrid Colicin E2-E3 using this approach and demonstrated that the expressed protein killed *E. coli* (not published). This technique provided us with the opportunity to produce our chimeric proteins in a more high-throughput manner.

To determine if this approach would be beneficial to our study, we expressed Colicin E2-Gp2 using *in vitro* transcription and translation. We added MG1655 *E. coli* to our protein production reaction and incubated the bacteria with the proteins during production. After incubation, we measured growth of bacteria using both a multi-well platform and by plating 2 μ L of reaction on to LB agar plates and incubating overnight at 37 °C.

As expected, we observed no growth of *E. coli* in reactions containing the positive control Colicin E2-E3 (Figure 7.10 A). We observed no growth attenuation in reactions containing Colicin E2-Gp2, demonstrating that the chimeric protein was not effective to inhibit growth of *E. coli*. Supporting these findings, we observed no growth of *E. coli* on LB agar plates for reactions containing Colicin E2-E3, however Colicin E2-Gp2 did not attenuate growth (Figure 7.10 B).

Whilst we did not see the desired growth attenuation of *E. coli* when treating with our chimeric protein, we determined the assay successful in that it provided a relatively quick way to produce our proteins, including the positive control Colicin E2-E3. We therefore used this assay for all protein production from this point forward and to test the activity of our chimeric proteins.

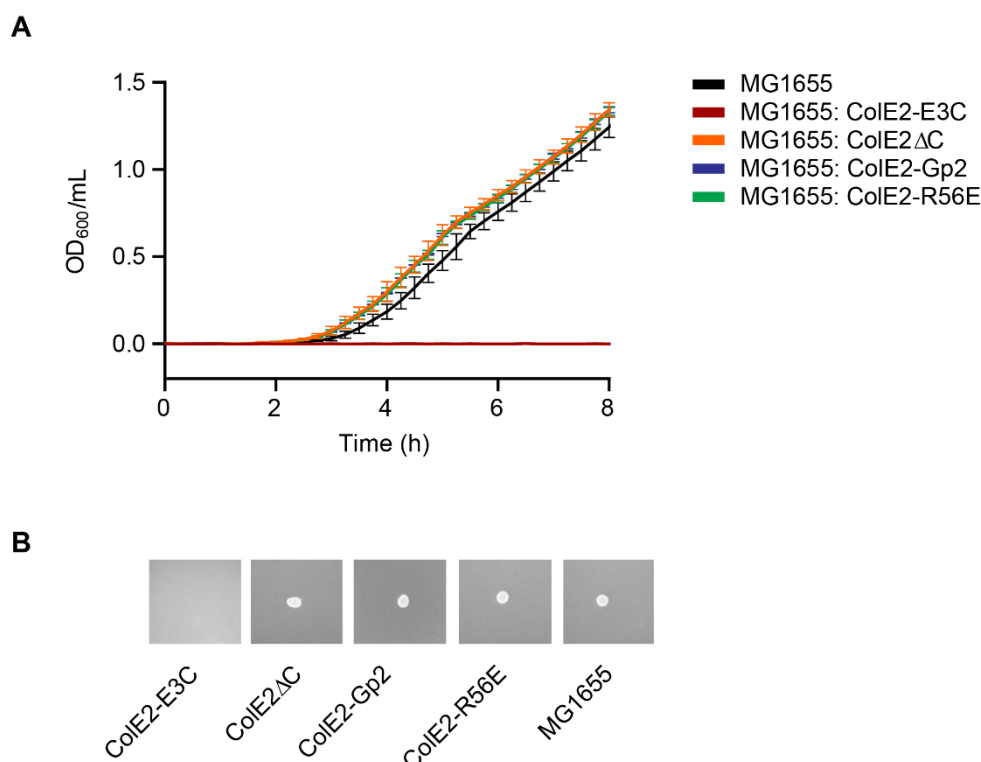


Figure 7.10: Exogenous Colicin E2-Gp2 does not inhibit bacterial growth

(A) *E. coli* were incubated with colicin constructs produced in a cell free *in vitro* transcription-translation assay. Bacteria were resuspended in LB and recovery growth of MG1655 *E. coli* measured by OD_{600nm} by (A) plate reader or (B) spotting 2 μ l of culture on to LB agar plates. Error bars represent standard deviation (n=3).

7.4.3. Redesign of chimeric constructs and experimental design

Our initial chimeric protein did not inhibit *E. coli* growth. Therefore, we proceeded to design a second set of constructs (Figure 7.11). Previously we had designed our chimeric protein to contain Gp2 with a small 2 amino acid linker after the colicin E2 receptor domain (Figure 7.5). However, the Colicin E2-E3 construct provided by Syngulon inserted the E3 cytotoxic domain directly after the colicin E2 receptor domain. We reasoned that this extra 2 amino acid linker may be affecting the activity of the colicin part of the chimeric protein. We have therefore designed further chimeric proteins placing our HAFs directly after the last residue of the receptor domain (Figure 7.11 B).

Our redesign process also considered the isoelectric charge of the proteins ²²⁴. The pI of a protein indicates whether a protein carries a net positive or negative charge ²²⁴. It has been shown in the literature that isoelectric charge of proteins plays a significant role in their transport across bacterial membranes ⁹. Whilst the mechanism by which cytotoxic domain of

colicins traverses the inner membrane of *E. coli* is poorly understood, it has been suggested that the pI and electrostatic charge of the cytotoxic domain may affect spontaneous insertion into the inner membrane and thus toxicity of the colicin ²²⁸. The pI of the cytotoxic domains of colicin E2 (9.75) and colicin E3 (9.79) are vastly different to that of Gp2 (5.32) (Figure 7.11A). With this in mind, we also designed a construct using Gp5.7 (Colicin E2-Gp5.7), another viral RNA polymerase inhibitor produced by T7 phage, with similar pI to colicin E2 and E3 cytotoxic domains (Figure 7.11 A).

As mentioned, the mechanism by which the colicin cytotoxic domain traverses the membrane is poorly understood. We reasoned that the cytotoxic domain itself may be important in facilitating transfer of a cargo protein across the membrane. Our third chimeric protein therefore retained the colicin cytotoxic domain, followed immediately by Gp2 (Colicin E2E3-Gp2). All construct designs can be seen in Figure 7.11 B, experiments will be separated by construct in the following sections, where we tested the ability of each construct to inhibit bacterial growth both in vivo and after treatment with exogenous protein.

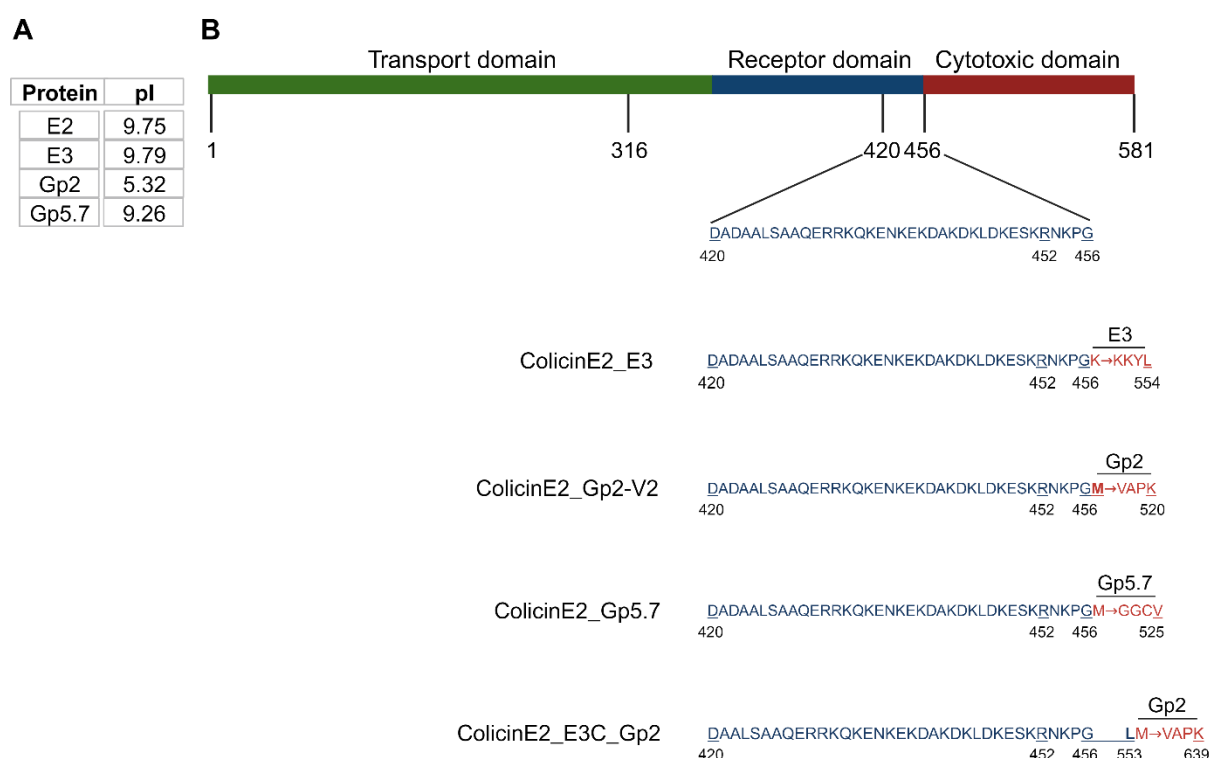


Figure 7.11: Domain structure of Colicin E2 and Colicin E2-hybrid protein designs

(A) Table shows isoelectric charge of proteins. pI refers to that of the cytotoxic domains of colicins E2 and E3. (B) Schematic displays the three domains of colicin E2 (transport, receptor and cytotoxic). Residues important for efficient cleavage (420, 452 and 456) are highlighted. Lower panel shows the design of each chimeric protein including residue numbers.

7.4.4. Colicin E2-Gp2 version 2

To determine the ability of the C-terminally His tagged Colicin E2-Gp2_V2 to attenuate bacterial growth, we cloned our chimeric protein construct into the arabinose inducible pBAD18 vector system. We performed growth inhibition assays using a multi-well platform in MG1655 *E. coli*. For induction from the araBAD promotor, we treated cultures with 0.2% v/v arabinose during exponential phase.

7.4.4.1. Colicin E2-Gp2 version 2 inhibits bacterial growth when expressed *in vivo*

The induction of Colicin E2-Gp2_V2 in exponentially growing *E. coli* resulted in fast attenuation of growth in MG1655 *E. coli* (Figure 7.12). This growth attenuation was similar to that caused by the wild type untagged Gp2. Colicin E2-R56E_V2 mutants did not inhibit growth in either strain, demonstrating that this is driven by the active Gp2 in the chimeric protein.

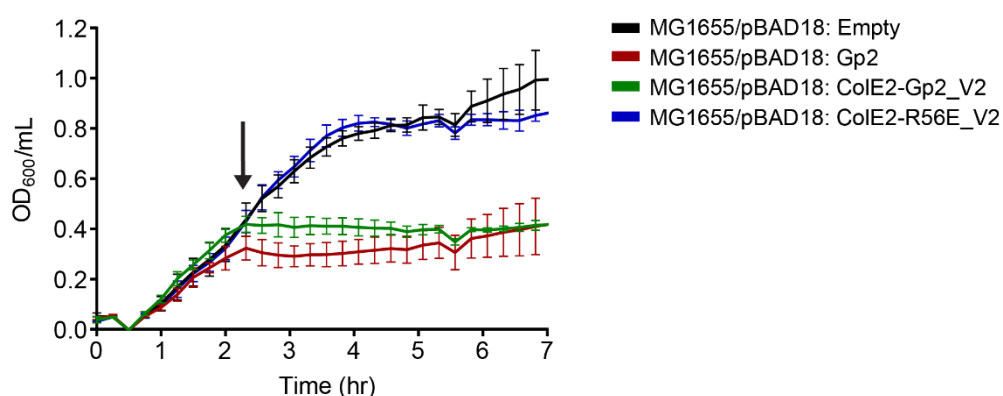


Figure 7.12: Colicin E2-Gp2 version 2 inhibits bacterial growth when expressed *in vivo*

Growth measured by OD600nm of MG1655 *E. coli* in LB. Gp2, Colicin E2-Gp2_V2 and Colicin E2-R56E_V2 were expressed from pBAD18 plasmids, arrows indicate induction of plasmids by addition of 0.2% L-arabinose at OD600 0.2. Error bars represent standard deviation (n=3).

7.4.4.2. Exogenous Colicin E2-Gp2 version 2 does not inhibit bacterial growth

To determine the delivery of Gp2 from our newly designed version 2, we expressed Colicin E2-Gp2_V2 using *in vitro* transcription and translation. We added MG1655 *E. coli* to our protein production reaction and allowed incubation of the proteins with the bacteria. After incubation, we measured growth of bacteria using both a multi-well platform and by plating 2 μ L of reaction on to LB agar plates and incubating overnight at 37 °C.

As with our previous construct, we observed no growth of *E. coli* in reactions containing the positive control Colicin E2-E3 (Figure 7.13 A); however, we observed no growth attenuation in reactions containing Colicin E2-Gp2_V2, demonstrating that Colicin E2-Gp2_V2 did not inhibiting growth of *E. coli*.

We also observed no growth of *E. coli* on LB agar plates for reactions containing Colicin E2-E3, however Colicin E2-Gp2_V2 did not attenuate growth (Figure 7.13 B).

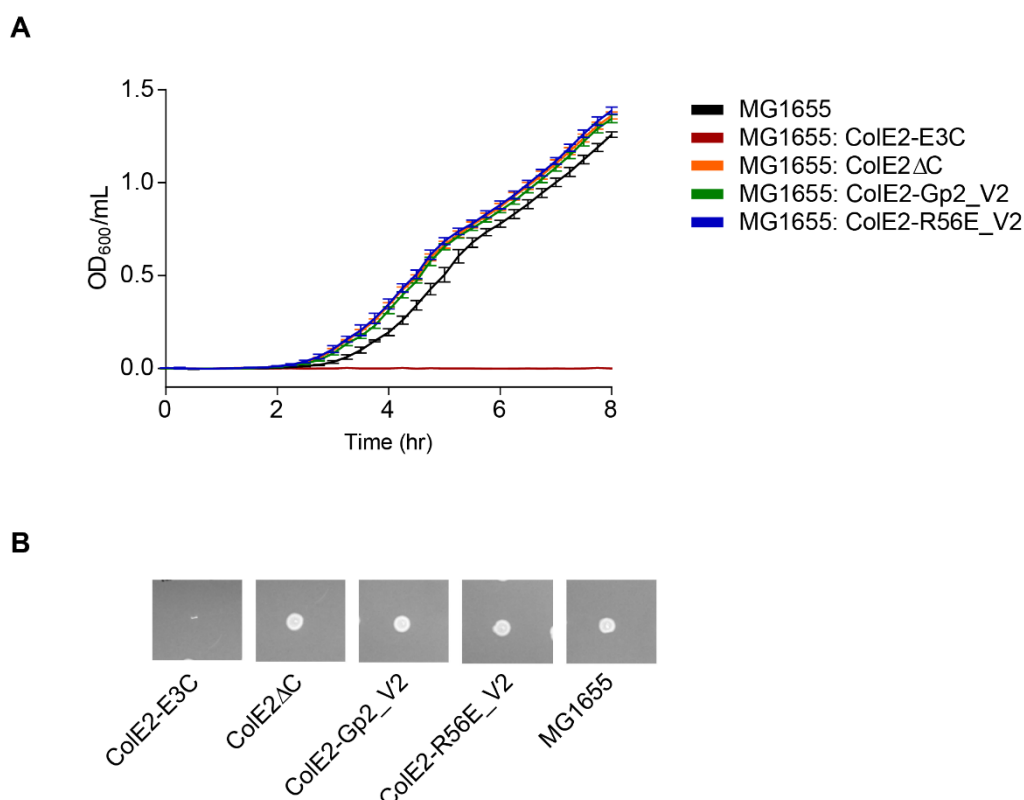


Figure 7.13: Exogenous Colicin E2-Gp2_V2 does not inhibit bacterial growth

(A) *E. coli* were incubated with colicin constructs produced in a cell free *in vitro* transcription-translation assay. Bacteria were resuspended in LB and recovery growth following resuspension was measured by OD_{600nm} by (A) plate reader or (B) spotting 2 μ l of culture on to LB agar plates. Error bars represent standard deviation (n=3).

7.4.5. Colicin E2-Gp5.7

7.4.5.1. Colicin E2-Gp5.7 inhibits bacterial growth when expressed *in vivo*

To determine the ability of our next construct, the C-terminally His tagged Colicin E2-Gp5.7, to attenuate bacterial growth, we performed growth inhibition assays using a multi-well

platform in MG1655 *E. coli*. For induction from the araBAD promotor, we treated cultures with 0.2% v/v arabinose during exponential phase.

The induction of Colicin E2-Gp5.7 in exponentially growing *E. coli* resulted in attenuation of growth in MG1655 *E. coli* (Figure 7.14). This growth attenuation was less efficient than that caused by the wild type untagged Gp5.7. Colicin E2-R24A mutants did not inhibit growth in either strain, proving that attenuation was driven by the active Gp5.7 in the chimeric protein. As expected from the literature²⁰¹, Gp5.7 was less effective at inhibiting RNAP and attenuating growth than Gp2. This data demonstrates that Gp5.7 can be C-terminally His tagged and still retain some of its inhibitory activity. We continued to investigate this protein despite the lower ability to attenuate growth in *E. coli*, as it would still provide an inhibitory read out for future assays.

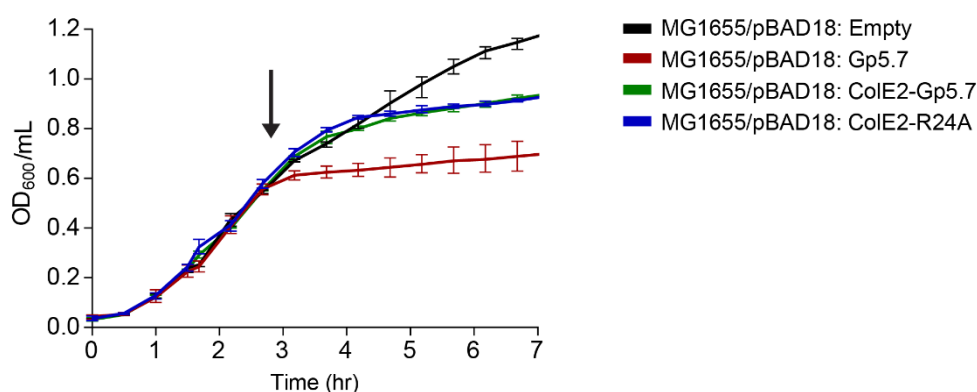


Figure 7.14: Colicin E2-Gp5.7 inhibits bacterial growth when expressed *in vivo*

Growth measured by OD600nm of MG1655 *E. coli* during N starvation. Gp2, Colicin E2-Gp5.7 and Colicin E2-R24A were expressed from pBAD18 plasmids, arrows indicate induction of plasmids by addition of 0.2% L-arabinose at OD600 0.2. Error bars represent standard deviation (n=3).

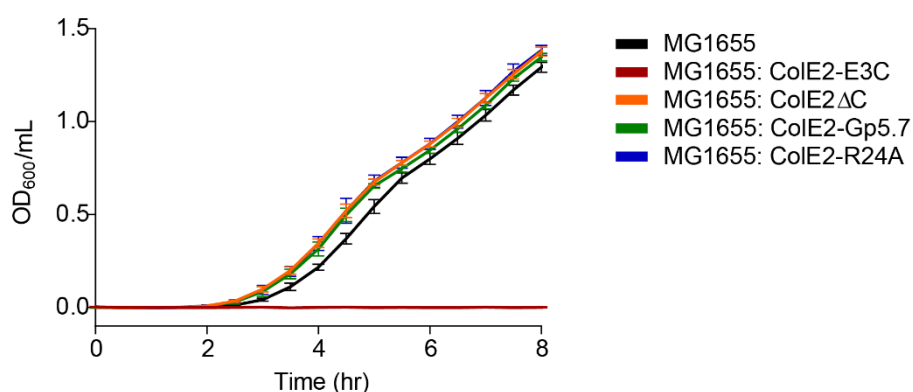
7.4.5.2. Exogenous Colicin E2-Gp5.7 does not inhibit bacterial growth

To determine if Gp5.7 could be delivered by Colicin E2, we expressed Colicin E2-Gp5.7 using *in vitro* transcription and translation. We added MG1655 *E. coli* to our protein production reaction and allowed incubation of the proteins with the bacteria. After incubation, we measured growth of bacteria using both a multi-well platform and by plating 2 μ L of reaction on to LB agar plates and incubating overnight at 37 °C.

As expected, we observed no growth of *E. coli* in reactions containing the positive control Colicin E2-E3 (Figure 7.15 A). We observed no growth attenuation in reactions containing Colicin E2-Gp5.7, demonstrating that Gp5.7 was not inhibiting growth of *E. coli*.

We observe no growth of *E. coli* on LB agar plates for reactions containing Colicin E2-E3, however Colicin E2-Gp5.7 did not attenuate growth (Figure 7.15 B).

A



B

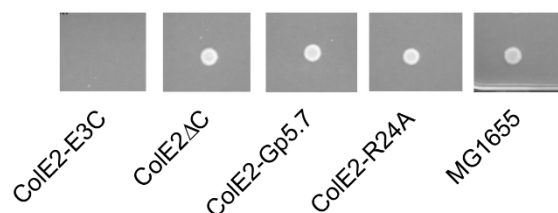


Figure 7.15: Exogenous Colicin E2E3-Gp5.7 does not inhibit bacterial growth

(A) *E. coli* were incubated with colicin constructs produced in a cell free *in vitro* transcription-translation assay. Bacteria were resuspended in LB and recovery growth following resuspension was measured by OD600nm by (A) plate reader or (B) spotting 2 μ l of culture on to LB agar plates. Error bars represent standard deviation (n=3).

7.4.6. Colicin E2-Gp2 and Colicin E2-Gp5.7 produced *in vitro* inhibit RNA polymerase activity

We did not observe any growth attenuation when treating *E. coli* with Colicin E2-Gp2_V2 or Colicin E2-Gp5.7. As we had utilised a new cell-free *in vitro* protein production method to produce these proteins, we reasoned that the proteins simply may not be active when

produced in this way. To rule this out, we took Colicin E2-Gp2_V2 and Colicin E2-Gp5.7 produced *in vitro* and determined their ability to inhibit RNAP, using the assay previously described in 2.4.2.3.

We observed that Colicin E2-Gp2_V2 inhibited at a dilution of 10:1, whilst Colicin E2-Gp5.7 inhibited at a dilution of 100:1 (Figure 7.16). We therefore concluded the Gp2 and Gp5.7 activity of both purified chimeric protein is retained when produced using cell free protein production.

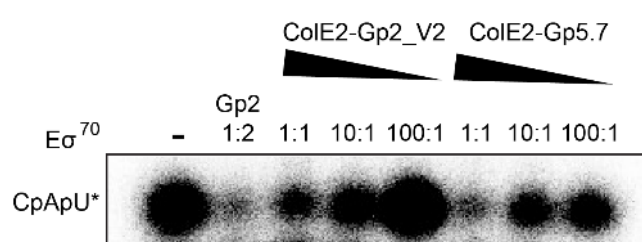


Figure 7.16: The effect of Colicin E2-Gp2_V2 and Colicin E2-Gp5.7 on *E. coli* RNA polymerase activity

Representative autoradiographs of denaturing urea gel, showing the ability of ColicinE2-Gp2 and ColicinE2-R56E to inhibit the ability of the *E. coli* RNAP holoenzyme ⁷ to synthesis dinucleotide primed RNA product from the T7 A1 promotor. Colicin E2-Gp2_V2 and Colicin E2-Gp5.7 used in this assay were produced in a cell free *in vitro* reaction. Radiolabelled nucleotides used in this assay are indicated by asterisk.

7.4.7. Colicin E2E3-Gp2

7.4.7.1. Colicin E2E3-Gp2 inhibits bacterial growth when expressed *in vivo*

To determine the ability of the C-terminally His tagged Colicin E2E3-Gp2 to attenuate bacterial growth, we cloned our chimeric protein construct into the arabinose inducible pBAD18 vector system. We performed growth inhibition assays using a multi-well platform in MG1655 *E. coli*. For induction from the araBAD promotor, we treated cultures with 0.2% v/v arabinose during exponential phase.

The induction of Colicin E2E3-Gp2 in exponentially growing *E. coli* resulted in fast attenuation of growth in MG1655 *E. coli* (Figure 7.17). This growth attenuation was similar to that caused

by the wild type untagged Gp2. We observed some inhibition of growth in Colicin E2E3-R56E, suggesting that the colicin E3 cytotoxic domain was having an effect on bacterial growth. This was somewhat unexpected, as Colicin E2E3-Gp2 and Colicin E2E3 caused similar growth inhibition. This data suggests that both Gp2 and the E3 cytotoxic domain affected bacterial growth.

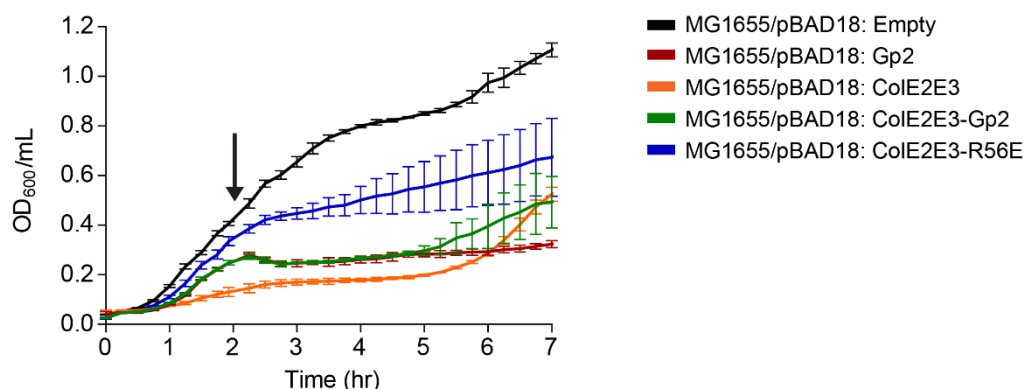


Figure 7.17: Colicin E2E3-Gp2 inhibits bacterial growth when expressed *in vivo*

Growth measured by OD_{600nm} of MG1655 *E. coli* during N starvation. Gp2, Colicin E2E3-Gp2 and Colicin E2E3-R56E were expressed from pBAD18 plasmids, arrows indicate induction of plasmids by addition of 0.2% L-arabinose at OD₆₀₀ 0.2. Error bars represent standard deviation (n=3).

7.4.7.2. Exogenous Colicin E2E3-Gp2 does not inhibit bacterial growth

In our previous chimeric protein designs, we replaced the cytotoxic domain of colicin E2 with a cargo protein (Gp2 or Gp.57). As previously discussed, the mechanism by which the C domain traverses the membrane is unknown, however it has been suggested in the literature that the C domain itself may play a role in insertion. We therefore reasoned that by adding Gp2 after the C domain, Gp2 may be able to ‘piggy back’ through the membrane with the natural C domain.

To determine if the colicin cytotoxic domain would facilitate delivery of Gp2, we expressed Colicin E2E3-Gp2 using *in vitro* transcription and translation. We added MG1655 *E. coli* to our protein production reaction and allowed incubation of the proteins with the bacteria. After incubation, we measured growth of bacteria using both a multi-well platform and by plating 2 μ L of reaction on to LB agar plates and incubating overnight at 37 °C.

As expected, we observed no growth of *E. coli* in reactions containing the positive control Colicin E2-E3 (Figure 7.18 A). We observed no growth attenuation in reactions containing Colicin E2E3-Gp2, demonstrating that Gp2 was not inhibiting growth of *E. coli*.

We observe no growth of *E. coli* on LB agar plates for reactions containing Colicin E2-E3, however Colicin E2E3-Gp2 did not attenuate growth (Figure 7.18 B).

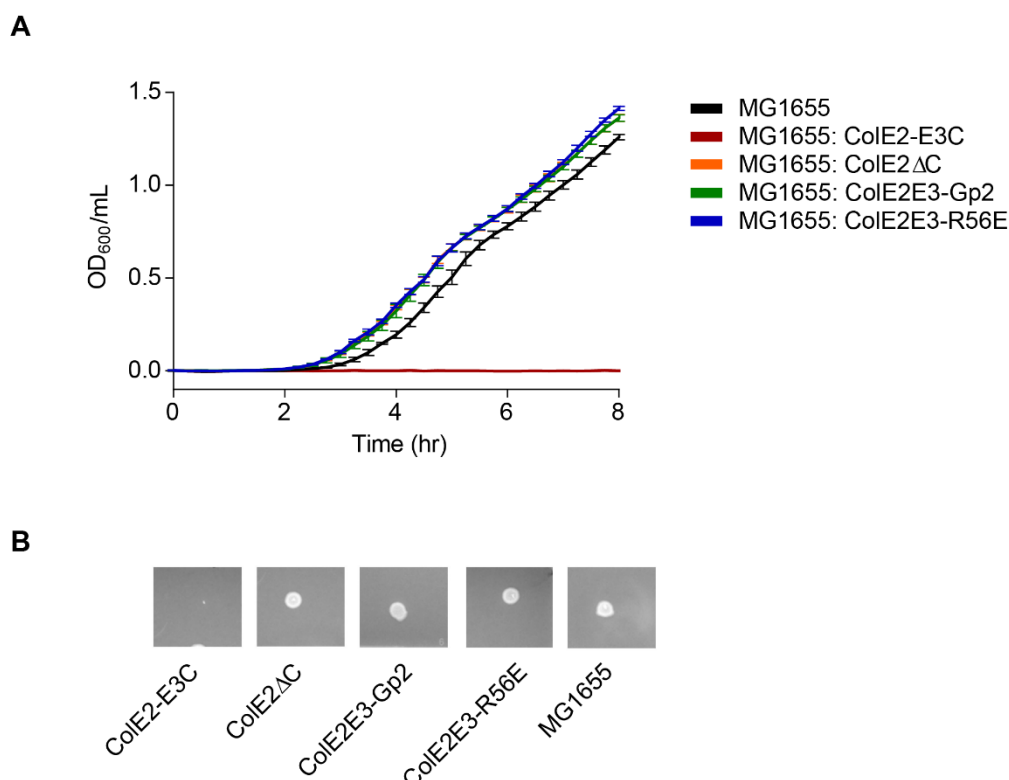


Figure 7.18: Exogenous Colicin E2E3-Gp2 does not inhibit bacterial growth

(A) *E. coli* were incubated with colicin constructs produced in a cell free *in vitro* transcription-translation assay. Bacteria were resuspended in LB and recovery growth following resuspension was measured by OD_{600nm} by (A) plate reader or (B) spotting 2 μ l of culture on to LB agar plates. Error bars represent standard deviation (n=3).

These data together show that intracellular expression of our chimeric colicin proteins inhibit bacterial growth. However, treatment with exogenous proteins were ineffective. We currently do not know where the design failed, this will be discussed below.

7.5. Chapter summary

- Intracellular expression of chimeric proteins of colicins and phage HAFs resulted in inhibition of bacterial growth
- Chimeric proteins purified using bacterial or cell free techniques retain activity of HAFs Gp2 and Gp5.7
- Addition of exogenous chimeric proteins does not result in inhibition of bacterial growth

7.6. Discussion

In this chapter, we attempted to deliver small T7 viral HAFs to *E. coli* using the colicin E2 as a delivery system. A major advantage of this approach was the potential of creating a 'plug and play' adaptable protein delivery system. This would allow us to utilise previously unexplored, naturally occurring small antibacterial proteins. Gp2 and Gp5.7 were chosen as RNAP transcriptional inhibitors, as the mechanism of action of these viral HAFs was well studied. Successful delivery of these HAFs would have resulted in growth attenuation in *E. coli*.

We did not observe any growth attenuation upon addition of any of our chimeric proteins. Below, we will discuss how and why delivery of active cargo could have failed.

During our initial experiments, we came across experimental design issues which we attempted to overcome. As discussed, colicin E2 is extremely toxic and difficult to purify by expressing in bacteria. We therefore conducted our initial exogenous protein experiments without a positive control. This meant we could not eliminate potential issues with our experimental setup, such as the denaturing of purified Colicin E2-Gp2 in LB at 37 °C. We also found our method of purification, via the AKTA-Pure and protein testing, was both cumbersome and ineffective. Going forward we decided to utilise cell free protein production to overcome these obstacles. This method was extremely useful to us, as it provided a way to produce our colicin E2-E3 positive control, and was a higher through-put method for testing new constructs.

We identified several stages where our chimeric proteins may fail to deliver their active cargo. These were; loss of activity of Gp2 when coupled to a large N terminal protein, lack of binding to *E. coli* BtuB receptors, inability to cross the bacterial cell wall, lack of cleavage by FtsH or lack of cargo activity once cleaved.

To determine if Gp2 was active when attached to colicin E2, we performed growth inhibition assays using a multi-well platform. We expressed the Colicin E2-Gp2 construct in *E. coli* using the arabinose inducible pBAD18 vector system. We observed expression of Colicin E2-Gp2 in *E. coli* resulted in growth attenuation, caused specifically by Gp2 (Figure 7.12). To date, we

have not explored the other stages where our chimeric protein delivery may have failed. We will discuss these stages next, along with future work which could be conducted .

Colicin E2 binds to the surface of *E. coli* via its receptor (R) domain, which facilitates high affinity binding to the BtuB receptor on target cells ^{196, 222}. Once bound, colicin E2 undergoes a conformational change, allowing it to release the immunity protein resulting in unfolding of the colicin before entering the target cell ^{196, 222}. It is possible that the activity of the R domain (and therefore binding capability of the chimeric protein) was disrupted by replacement of the colicin E2 C-domain with Gp2, possibly by changing the conformation of the protein. Interestingly, our chimeric protein Colicin E2E3-Gp2 did not inhibit bacterial growth of *E. coli* when produced via our cell free assay (Figure 7.18). This showed that addition of Gp2 inactivated Colicin E2E3 in some way, for example by preventing binding to the target cell or through inactivation of cytotoxic domain E3. To date we have not determined the cause of this inactivation. As discussed, colicin E2 must unfold before enters the cell. Gp2 and Gp5.7 are folded and active when produced via the AKTA pure and our cell free transcription-translation assay. We therefore speculated that after binding of our chimeric protein to target *E. coli*, Gp2 may simply sit at the cell surface in its folded state without a mechanism to unfold, which prevented it from entering the target cell. It may therefore have been better to use colicin E2 to deliver small nucleic acids in the first instance, as the effect of tertiary structure of these would not be as great due to their size, shape and composition.

The next stage where delivery may have failed was crossing the bacterial cell wall. The literature shows that after colicin E2 binds bacteria via its R-domain, the protein undergoes a conformational change which releases the immunity protein ^{196, 222}. The bound complex recruits OmpF proteins which facilitates transport of the T-domain into the periplasmic space (Figure 7.3). It is common that the size and charge of a protein are extremely important factors which determine interactions of proteins with transport channels ²²⁹, therefore our chimeric protein may simply not be the correct size, composition of amino acids and charge to facilitate entering the OmpF channel. The transport domain interacts with the TolA and TolB proteins in the periplasmic space (Figure 7.3). It is unknown how the C-domain passes through the inner membrane, although it has been suggested that the T domain facilitates transport of the cytotoxic domain to the inner membrane. This mechanism may be disrupted by Gp2 and Gp5.7, or these proteins may not have the right biochemical properties to be transported in the same way as the colicin C-domain. If any of these interactions are disrupted, it is highly likely that the cargo protein would not be delivered to the cytoplasm.

The final stage where our chimeric protein may have failed was lack of cleavage by FtsH at the inner membrane or lack of cargo activity once delivered to the cytoplasm. It may be that

Gp2 and Gp5.7 were delivered through the outer membrane and periplasmic space, but simply were not being cleaved by FtsH to release them at the inner membrane. As discussed, once the C-domain has traversed the inner membrane, it is cleaved at residue 420 by the cytoplasmic inner membrane protein FtsH (Figure 7.4 G) ²²³. We included residue 420 in our chimeric protein design (Figure 7.11) to try to retain cleavage activity and to determine if the chimeric proteins were able to be cleaved. Future work could express the proteins in *E. coli*, and carry out a western blot against Gp2 and Gp5.7. Depending on the size of the proteins on the gel, we would determine if the cargo were still attached to the other colicin domains and confirm if our chimeric proteins were still cleavable by FtsH. It is also possible that Gp2 and Gp5.7 may be unable to be refolded correctly inside the cell. As discussed, these cargo proteins would likely need to be unfolded to traverse the cell wall, meaning refolding was necessary once delivered to the cytoplasm. Whilst we were able to show that chimeric colicins produced via cell free methods retained their Gp2 and Gp5.7 functionality (Figure 7.16), it may be that Gp2 and Gp5.7 were inactive after transport through the cell membranes.

Many of the above ideas could be investigated using similar techniques, which would allow us to visualise the progress of our chimeric proteins. Future work would include a cell fractionation of treated bacteria to determine if the chimeric proteins were binding to the target bacteria, if any Gp2 or Gp5.7 was delivered to the cytoplasm and if these cargos were cleaved by FtsH. This would allow us to separate the cell membranes from the cytoplasm, and determine the location of the cargo proteins via western blot. Another method to track our proteins would be fluorescently tagging Gp2 and Gp5.7 with Alexafluor dye. This would allow us to visualise the localisation of the cargo proteins during our experiments. It is possible to block the BtuB using vitamin B, this would allow us to determine if the construct binds to the cell via the receptor.

As discussed, the structure and charge of proteins affects their interactions with other proteins and lipids, including membrane porins and cellular membranes. We attempted to take this into account when redesigning our chimeric proteins, opting to utilise a HAF with a pI more similar to colicin E3 C-domain (Figure 7.11). It therefore may be useful to design a simplified chimeric protein which allows us to easily assess delivery. One such way of doing this would be to replace the C-domain of our chimeric protein with split-GFP. Split GFP consists of two fragments of GFP, which only fluoresce after interacting with their counterpart, one of which is produced within the cell ²³⁰. A construct such as this would allow us to easily determine if the cargo had been delivered, as the GFP would only fluoresce if the cargo was delivered into the cytoplasm.

Ultimately, we moved on from this project when another group delivered DNA using colicins ²³¹. Whilst this publication provided proof of concept that colicins could be used as a delivery

mechanism, it somewhat removed the novelty of our work. Due to the pandemic, we were unable to take full advantage of Syngulon's expertise in the field of bacteriocins. This, coupled with the interesting progress made in the second project, lead to the decision to discontinue this project.

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8. Appendices

8.1. Appendix 1

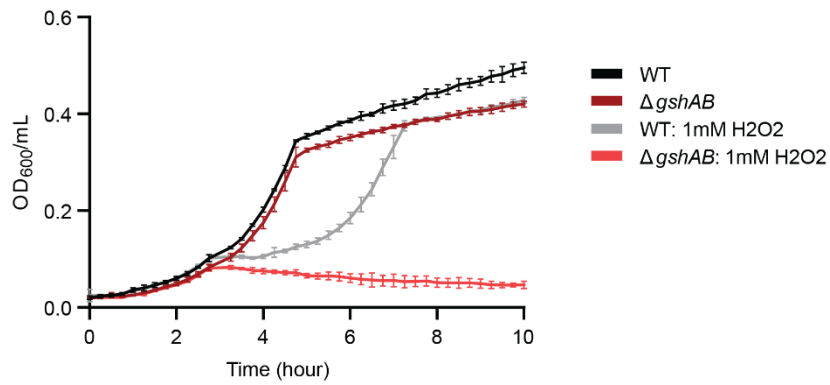


Figure 8.1: Addition of H₂O₂ causes growth attenuation of $\Delta gshAB$ bacteria during exponential growth in N starvation Growth measured by OD₆₀₀nm of *E. coli* MG1655 during N starvation. 1 mM H₂O₂ was added to cultures during exponential phase (approximately 3 hours).