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An investigation into an *in vitro* model of post-death metabolism and the cryptic growth phase.

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Thesis submitted for the degree of Master of Science by Research

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Durham University

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<u>Abstract</u>

Bacteria in nature are normally found in the long-term stationary phase due to the nutrient poor environments they grow in. This fifth phase has traditionally been neglected by microbiologists despite its importance in understanding how wild bacteria behave. It is known that the long-term stationary phase depends on cryptic growth – a process where material from dead bacteria is recycled as nutrients for the remaining living bacteria. It allows a colony to survive despite the lack of available nutrients. Whilst cryptic growth has been accepted in the literature since the late 1950's, no previous attempt has been made at understanding the precise mechanisms which underpin it. To start to understand this essential process, an *Escherichia coli* mutant with the Lon protease knocked-out was investigated through short and long-term growth assays. The Lon gene had previously been shown to cause a boost in the growth of a culture when present in its extracellular environment. This thesis presents evidence for the post-death activity of the Lon protease.

The absence of the Lon protease was observed to alter the growth phases of the *E. coli* population. The Lon-deficient cultures failed to enter the stationary phase promptly and, significantly, no evidence was seen of cryptic growth or entry to the long-term stationary phase. Additionally, the results show that Lon knock-outs have a temporary fitness advantage when in a mixed wild-type mutant culture, especially when at a low initial concentration, although this is unlikely to be selected for. In conclusion, Lon plays a fundamental role in post-death metabolism and also potentially its regulation. This puts the role Lon plays in the cell into a new perspective

and may lead other catabolic enzymes to be considered for complementary roles in

post-death metabolism.

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__Abbreviations__

Amp^R Ampicillin Resistance

AAA$^+$ ATPases associated with diverse cellular activities

BCA   Bicinchoninic Acid

BSA   Bovine Serum Albumin

CI   Confidence Interval

Cm^R Chloramphenicol Resistance

CFU   Colony Forming Units

FITC   Fluorescein isothiocyanate

GASP   Growth Advantage in Stationary Phase

Kam^R Kanamycin Resistance

LB   Luria-Bertani

LGA   Long-term Growth Assay

OD   Optical Density

ns   Not Significant

rcf   Relative Centrifugal Force

RFU   Relative Fluorescent Units

SD   Standard Deviation

SDS-PAGE   Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

S.E.M   Standard Error of the Mean

WT   Wild Type

Statement of Copyright

Declaration

I declare that this thesis for the degree of Master of Science by research has been composed solely by myself, and that no part has been submitted in any previous application for a degree. Except where stated otherwise by acknowledgement or citation, the work presented here is entirely my own. Where experimental work performed by others is shown, this is done with permission from S.E.R Gibson and Prof. M. Cann.

Acknowledgements

There are a large number of people whom without this project would not have been possible. Firstly, I would like to thank Martin Cann for this fabulous opportunity. I am so grateful for his continuous advice and guidance throughout this year and for pushing me to achieve results I never thought I could.

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I was incredibly lucky to end up in such a kind and welcoming lab group and I've massively benefitted from all the help and knowledge they were kind enough to give me. Thank you especially to Savannah for being my mother duck and teaching me everything I needed to know about this project and giving me more horrifying fun facts about bacteria and TB than I could ever need! Laura, Ella, and Amber – it was a joy both to work with and attempt to the daily crossword with you. I wish you all the best.

Finally, to all my friends and family; I could not have done this without your constant encouragement and belief in me. Thank you for letting me ramble about my 'zombie bacteria' and for at least pretending to understand! I am forever grateful for your support.

1. Introduction

1.1 Bacterial Growth Phases

1.1.2 Classical depiction.

The classical - and often taught - version of a bacterial growth cycle includes four phases: the lag phase, the log phase, the stationary phase, and the death phase (Maier and Pepper, 2015). Transitions between these phases often include both morphological and physiological changes in the cell, such as changes to DNA structure, size of the cell, and protein expression (Sobetzko, Travers and Muskhelishvili, 2012; Chubukov and Sauer, 2014; Jaishankar and Srivastava, 2017). During such a cycle, all usable nutrients in the surrounding area will be depleted, leading to the complete death of the culture. Should more nutrients be added, however, the culture can start to regrow hence it is named the feast-famine cycle. However, this model is incomplete and inaccurate to how bacteria in nature are found.

1.1.2 The long-term stationary phase.

This simplified model fails to mention a fifth phase of bacterial growth called the long-term stationary phase. It has also been called the phase of decline or the period of prolonged decrease (Gay, 1935; Monod, 1949). Figure 1 illustrates this complete model of bacterial growth. This phase is very similar to the first stationary phase. It starts after the loss of the majority (between 90 and 99%) of the culture's viable cells during the death phase (Navarro Llorens, Tormo and Martínez-García, 2010). *Escherichia coli* can survive in this phase for over a year in laboratory conditions without the addition of nutrients (Finkel and Kolter, 1999). The long-term stationary

phase is far more similar to how bacteria are found in nature, where the lack of available nutrients forces around 60% of bacteria to stay in this stationary/quiescent phase – at least until additional resources are added (Kolter, Siegele and Tormo, 1993; Navarro Llorens, Tormo and Martínez-García, 2010).

There is a temptation to assume that the long-term stationary phase is static, where the population is simply being maintained. This, however, is not the case. The population is dynamic, with a high mutation rate leading to higher genetic variability within the cultures. Mutants start to appear in the population whose specific traits make them better suited for survival under these conditions (Zambrano *et al.*, 1993; Navarro Llorens, Tormo and Martínez-García, 2010). Many of these mutations are related to a cell's ability to scavenge nutrients from its environment. These traits are called Growth Advantage in the Stationary Phase (GASP) phenotypes. GASP phenotypes are also characterised by their ability to outcompete both non-mutated cells and any previous generations of mutants. This leads to constant cycling of the population (Zambrano and Kolter, 1996; Finkel, 2006; Katz *et al.*, 2021). Several well-written reviews have been published on this topic (Zambrano and Kolter, 1996; Zinser and Kolter, 2004; Finkel, 2006).

The most common mutation leading to a GASP phenotype is a mutation in *rpoS,* which produces the sigma factor σ^S. It is responsible for activating the transcription of genes relating to stress responses and can affect around 10% of the *E. coli* genome (Hengge, 2008). This is often the first mutation, although other GASP phenotypes can arise independently (Martínez-García, Tormo and Navarro-Lloréns, 2003).
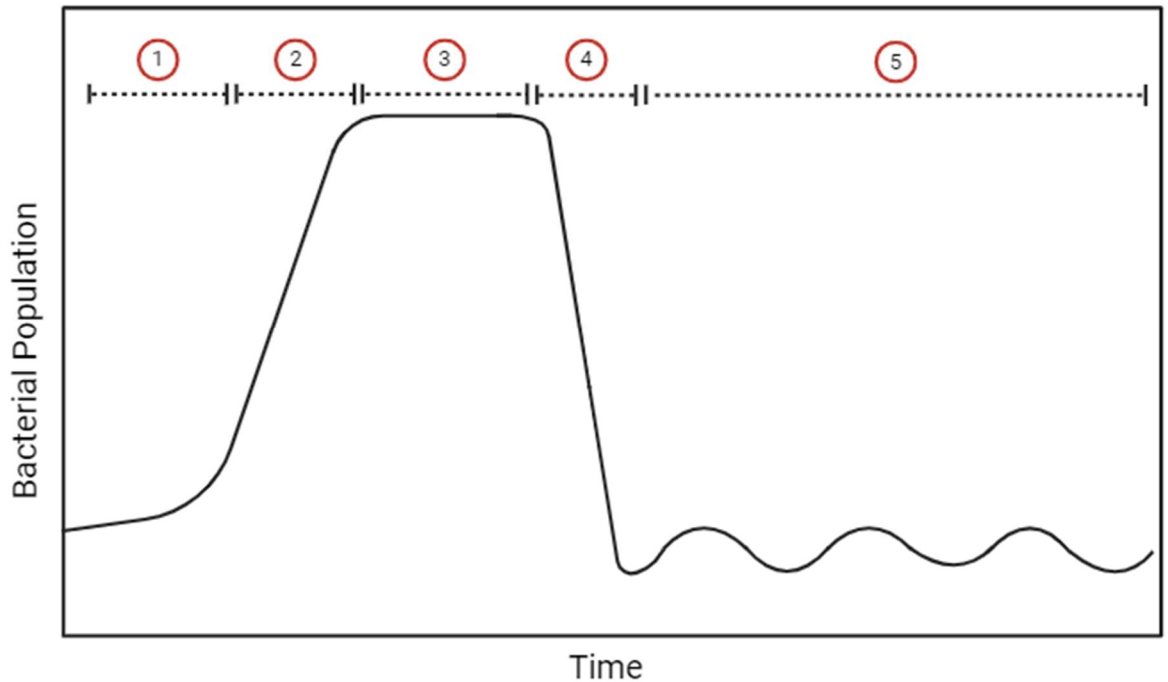
Figure 1: Growth curve illustrating the five main phases of bacterial growth. 1: the lag phase. 2: the log phase. 3: the stationary phase. 4: the death phase. 5: the long-term stationary phase. During the long-term stationary phase, the population is dynamic both in number of bacteria and in composition. Figure created with BioRender.com.

1.1.3 Cryptic growth / Post-death metabolism.

Bacterial survival under nutrient stress in the long-term stationary phase is due to several key responses. The stringent response is where there is a metabolic shift in the living cells, encouraging protein turnover and reducing the number of novel biomolecules created. It also reduces the production and increases the regulation of RNA synthesis (Mason, 1986).

The second response is a phenomenon called cryptic growth. Cryptic growth is the utilisation of nutrients provided by dead lysed bacteria in the same population (Mason and Hamer, 1987; Finkel, 2006). It was first theorised in 1959 and shown to exist in *E. coli* cultures the same year. It has since been demonstrated with other species, such as *Klebsiella pneumoniae* (Koch, 1959; Ryan, 1959; Mason and Hamer, 1987). Not much, if anything, has been written in the literature about the

mechanisms that underpin cryptic growth, although steps have been made with recent work by the Cann group (Gibson and Cann 2022, Personal Communication). In this study, the activity that underlies cryptic growth has been termed post-death metabolism.

1.2 The discovery of a mechanism underlying post-death metabolism

The Cann group identified a protein involved in facilitating post-death metabolism. First, they confirmed that the effects of post-death metabolism are visible in *E. coli* cultures when under nutrient stress. BW25113 *E. coli* were first grown in M9 minimal media, which put them under significant nutrient stress. The culture was knocked-back to an OD_{600} of 0.05 before undergoing a second round of growth in the presence of a lysate made from a previous BW25113 culture also under nutrient stress. The BW25113 cells grown in the presence of the BW25113 lysate significantly outgrew the cells which were supplemented with water (data not shown). Therefore, the BW25113 lysate provided a large growth boost and showed that growing cells could use nutrients from the extracellular environment for their benefit.

The above experiment was repeated with various combinations of BW25113 and BL21 growing strains and their respective lysates. The BW25113 strain is the Keio collections' parent strain, whilst BL21 is a strain commonly used for protein purification and biotechnology (Grenier *et al.*, 2014; Jeong, Kim and Lee, 2015). The significant difference between the two strains is the absence of several key proteases in the BL21 strain, which helps reduce protein degradation during production and purification (Jeong, Kim and Lee, 2015). The presence of the BW25113 lysate, regardless of what growing strain was used, was required to see the growth benefit

compared to the growing strain supplemented with water. The growth boost was not

seen when the BL21 lysate was used (data not shown).
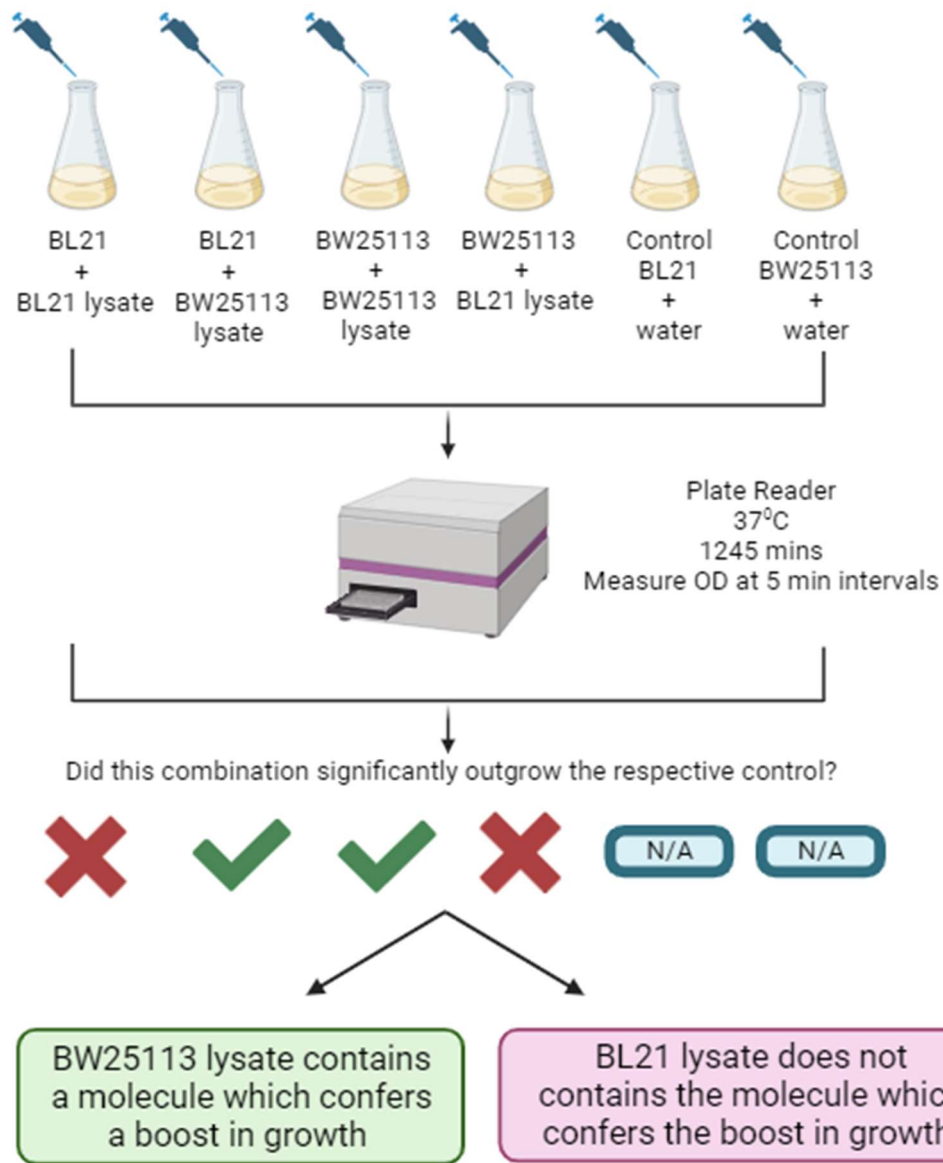
Figure 2: Diagram illustrating the methodology used to discover the growth boost effect of the BW25113 lysate. In subsequent experiments different combinations of BW25113, BW25113Δ*lon* and BW25113Δ*ompT* and their respective lysates were also tested using the same methodology. Figure made with Biorender.com

The BL21 strain is missing the genes for two key proteases – *lon* and *ompT* – which

BW25113 does have (Jeong, Kim and Lee, 2015). Lysates of the BW25113Δ*lon* (Δ*lon*)

and BW25113Δ*ompT* (Δ*ompT*) strains from the Keio collection were then tested using BW25113 as the growing strain. The growth boost was seen with the Δ*ompT* strain alone (data not shown). Figure 3a shows the results of a growth curve demonstrating that the absence of Lon in the lysate leads to a lack of growth compared to when it is present. It also demonstrates that this effect is reversed when a lysate from a rescued Δ*lon* is used (BW25113 Δ*lon*:Lon WT; Δ*lon*R). Figure 3b shows a similar experiment, but using the Δ*lon* strain as the growing strain, and shows conclusively that it is the presence of the Lon in the extracellular environment which causes any growth advantage. It also shows that a Lon protease with its protease activity knocked-out is also incapable of conferring a boost in growth. This data shows that, at least in this simulated environment, the presence of Lon is essential for cryptic growth and post-death metabolism. As the growth effect was present when the BW25113 lysate was used, regardless of whether the growing strain was deficient of Lon, it can be ruled out that the growth boost is due to the internal recycling of free ribosomal proteins, which has been previously shown to occur (Tsilibaris, Maenhaut-Michel and Van Melderen, 2006).
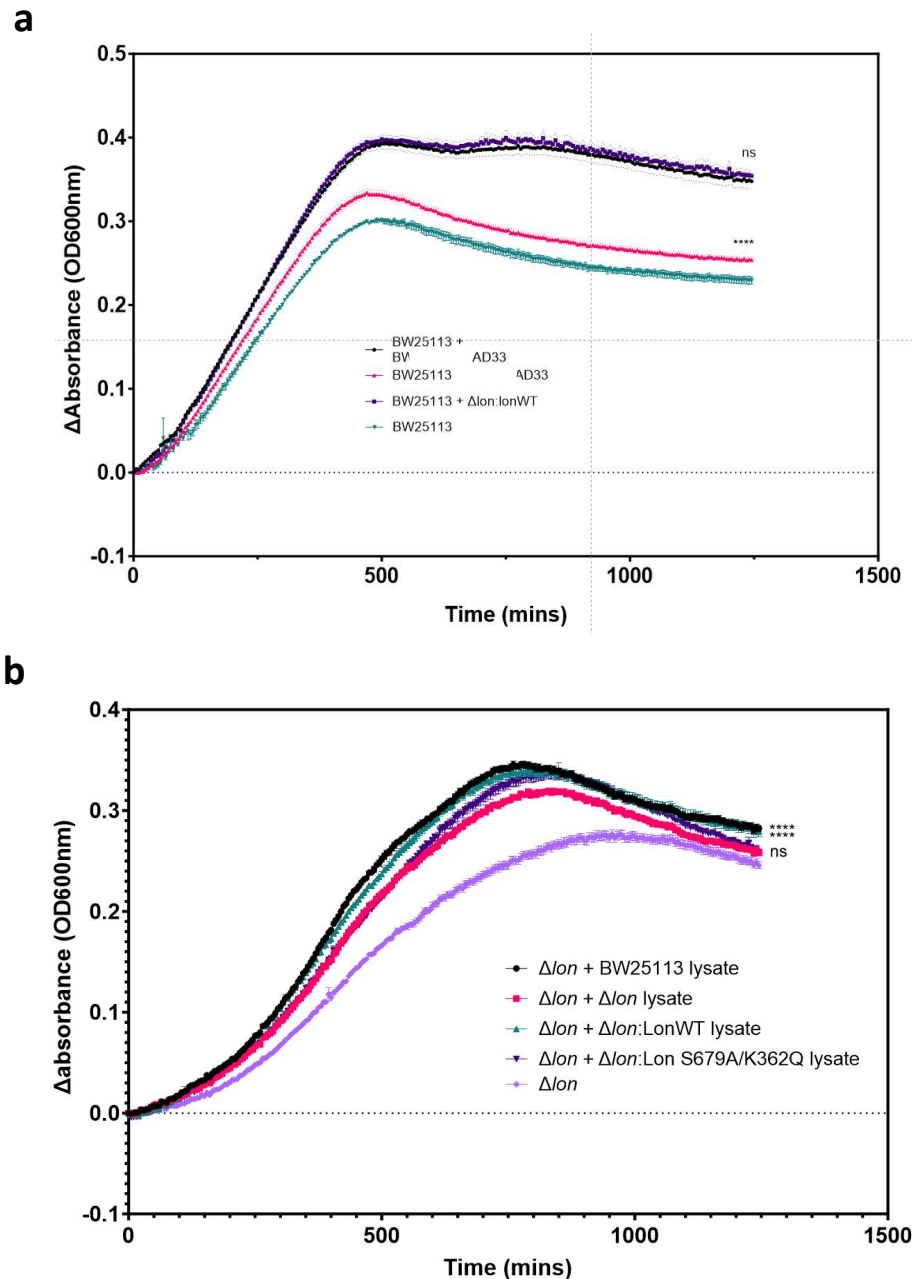
Figure 3: Growth curves comparing BW25113 (a) and Δ*lon* (b) growing strains supplemented with BW25113, Δ*lon* and Δ*lon*:LonWT lysates. a) Growth Curves comparing the growth of BW25113 cells (green) to BW25113 plus BW25113 lysate (black), BW25113 plus BW25113Δ*lon* lysate (pink) and BW25113 with BW25113Δ*lon*:LonWT lysate (purple). Cells were grown for 1245 mins and growth is indicated by change in OD_{600}. Stars represent level of significance (P < 0.0001; ns > 0.05) between endpoint OD values of BW25113 plus BW25113 lysate and other combinations as determined through a one-way ANOVA. **b**) Growth Curves comparing the growth of Δ*lon* cells (light purple) to Δ*lon* plus BW25113 lysate (black), Δ*lon* plus Δ*lon* lysate (pink), Δ*lon* with Δ*lon*:LonWT lysate (green) and Δ*lon* plus Δ*lon*:Lon S679A/K362Q lysate. Cells were grown for 1245 mins and growth is indicated by change in OD_{600}. Stars represent level of significance (P < 0.0001; ns > 0.05) between endpoint OD values of Δ*lon* with no lysate and the other combinations as determined through a one-way ANOVA. Error bars show standard error of mean (S.E.M) where n = 6. Data used with permission of S.E.R Gibson.

E. coli cells have an uptake limit of approximately five amino acids or 650 Da (Payne and Gilvarg, 1968). Peptides are imported through one of three permeases*:* the oligopeptide permease, the dipeptide permease and the tripeptide permease (Manson *et al.*, 1986). The oligopeptide permease can only import chains between three and five peptides (Guyer, Morgan and Staros, 1986).

It was theorised that Lon is released from the lysed cells and degrades the protein content of the lysate into small enough chains to be taken up by the living cells. Without Lon being released into the extracellular environment (or present in the cells before being lysed), the protein content cannot be broken down into small enough peptides.

1.3 The Lon Protease

Lon is a very heavily conserved 525 kDa protease, which is well described in the literature   (Lee and Suzuki, 2008; Van Melderen and Aertsen, 2009; Ellen F Vieux *et al.*, 2013). There are two subfamilies of Lon: Lon A and Lon B. The Lon B subfamily predominantly contains the forms found in archaeal species. Lon A is the form found in most of bacterial and eukaryotic species, including *E. coli* (Botos *et al.*, 2019)*.*

1.3.1 Biological functions of Lon.

The *E. coli* protease Lon (or La as it is sometimes known) was first discovered as a heat shock protein in 1984   (Chung and Goldberg, 1981; Phillips, VanBogelen and Neidhardt, 1984). Heat shock proteins are a large family of proteins expressed in response to heat stress, amongst other forms. As a chaperone-protease, Lon helps ensure that proteins are folded correctly after translation and when the cell is stressed. Heat-shock in *E. coli* occurs when the temperature reaches above 37°C,

causing its proteins to denature. Other stressors can also elict the same reaction as well (Maurizi, 1992). These denatured proteins are then tagged for degradation to prevent damage to the cell (Meyer and Baker, 2011). 70-80% of these denatured proteins are degraded by either the Lon or ClpXP proteases.

The Lon protease is unusual as aside from its role as a chaperone-protease, it also has a regulatory role to play in stress. For example, it degrades a regulatory protein called GadE, which controls the acid stress response. The degradation of GadE by Lon prevents the acid stress response when it is no longer needed (Heuveling, Possling and Hengge, 2008).

The SOS response is another stress response invoked in bacteria undergoing DNA damage. The response inhibits cell division and upregulates and activates DNA repair mechanisms (Janion, 2008). DNA can become damaged for many reasons, including high pressure, ultraviolet radiation and reactive oxygen species (Aertsen and Michiels, 2005; Baharoglu and Mazel, 2014). It has been proposed that there is a link between nutrient stress and the SOS response, as the SOS response and carbon source stress are regulated through the same cAMP-CRP complex (Baharoglu and Mazel, 2014). However, this has not yet been demonstrated. The Lon protease regulates the SOS response at two main levels. It degrades the LexA repressor, allowing, the transcription of the SOS-activated genes (Pruteanu and Baker, 2009). It also degrades SulA, which inhibits cell division, ensuring that once the DNA damage has been mitigated, cell division can proceed again (Mizusawa and Gottesman, 1983).

1.3.2 The structure of the Lon protease.

The Lon polypeptide chain is 784 amino acids long and consists of three domains: the N-terminal substrate recognition domain, the ATPase domain, and the C-terminal proteolytic domain. The presence of the ATPase domain is characteristic of the AAA$^+$ family of proteins – ATPases associated with diverse cellular activities (Striebel, Kress and Weber-Ban, 2009; Puchades, Sandate and Lander, 2020). The substrate recognition domain is highly variable across species, but the precise mechanics of how it recognises its targets are not fully known (Tsilibaris, Maenhaut-Michel and Van Melderen, 2006; Brown *et al.*, 2019). Some have suggested that a domain between the ATPase and C-terminal may additionally be involved (Tsilibaris, Maenhaut-Michel and Van Melderen, 2006). Lon differs from several other AAA$^+$ proteases, such as ClpXP, because the ATPase, protease, and regulatory domains are physically connected on the same polypeptide chain rather than being different subunits which are then assembled (Gottesman, 1996). However, it still contains the evolutionarily conserved Walker A motif to which ATP binds (Fischer and Glockshuber, 1994).

Despite this difference, the Lon monomers assemble into a classic, barrel-shaped, homo-hexameric structure (figure 4). Two Lon hexamers can interact at the N-termini and assemble to form a dodecamer (Ellen F Vieux *et al.*, 2013). There is currently no consensus on how the two hexamers interact; Brown *et al.*, 2019 suggest that the interaction is head-to-head whilst others suggest an asymmetric V-shaped structure (Botos *et al.*, 2019). It is
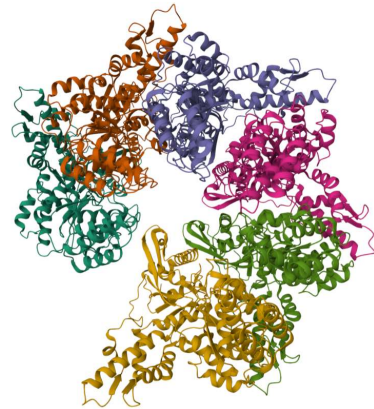
Figure 4: Cryo-EM image of *E. coli* Lon protease (Botos *et al.*, 2019)**.** Image created from PDB (6U5Z) (Berman *et al.*, 2000; Rose *et al.*, 2018). Each protomer in the hexamer is shown in a different colour.

known, however, that the ratio of hexameric vs. dodecameric Lon may be altered in response to stress, in addition to the gene being upregulated (Ellen F Vieux *et al.*, 2013). This dodecameric structure prevents the degradation of large protein substrates but not any which are small enough to fit through the equatorial poles (Ellen F. Vieux *et al.*, 2013; Kirthika *et al.*, 2023).

1.3.3 The mechanisms of Lon-mediated protein degradation.

The mechanism by which Lon recognises its substrates has not been fully elucidated. Lon recognises degradons, short peptide sequences that should only be exposed if the protein target is incorrectly folded. The binding of Lon at its N-termini to the degradon is known to increase its protease activity fivefold (Wohlever, Baker and Sauer, 2014). The exact sequences Lon recognises are variable, not well defined and can be found at either the end of a polypeptide chain or in an exposed loop. This variance is essential to Lon's role as a promiscuous protease as it allows for a wide

range of proteins to be targeted for proteolysis if required (Ishii *et al.*, 2000; Gur and Sauer, 2008). However, a common trait of the degradons is that they are often highly hydrophobic (Li *et al.*, 2021). The binding of the substrate to the N-terminal also causes the protomers to shift from an open to a closed configuration (Botos *et al.*, 2004).

Substrate binding confers an ATP-dependant conformational change to the position of the N-terminus. It moves closer to the protease domains to deliver the substrate (Lin *et al.*, 2016; Tzeng *et al.*, 2021). The proteolytic domains are in the central channel, which is 18 Å in diameter and so can accommodate an α-helix or β-fold inside it, but no larger structures (Botos *et al.*, 2004). Therefore, for the substrate to fit and be degraded, it must first be unfolded – a process also dependent on the energy released by hydrolyses of ATP. In contrast, hydrolysis of a peptide bond requires no energy input. The active site of Lon is made from a serine-lysine catalytic dyad (Amerik AYu *et al.*, 1991; Botos *et al.*, 2004). In *E. coli,* these are Ser679 and Lys722 (Rotanova *et al.*, 2006). Whilst this is a common catalytic pairing, the *E. coli* Lon dyad is found in a unique fold structure. The nucleophilic hydroxyl group on the serine attacks the peptide bond, hydrolysing it. There are also indications that other amino acids, such as a conserved threonine (Thr704 in *E. coli*) are involved in stabilization of the intermediates (Botos *et al.*, 2004; Rotanova *et al.*, 2006). The final result of Lon-mediated proteolysis is peptides of around five amino acids in length, although multiple cycles may be required for complete degradation (Mikita *et al.*, 2013).

The dodecamer conformation has been shown to have a lower activity for larger substrates but an approximately equal activity for smaller proteins/peptide chains. This may be because the binding sites between the two hexamers block some substrate recognition sites. This leads to the Lon dodecamer relying exclusively on the size and hydrophobicity of the protein to determine which substrates can reach the inside, or lumen, of the dodecamer and be degraded. As a result, the dodecamer structure has a lower ATP requirement (Ellen F Vieux *et al.*, 2013).

Despite not being studied heavily, there are at least two reports of *E. coli* Lon having baseline ATP-independent activity (Maurizi, 1987; Fischer and Glockshuber, 1994). Additionally, LonB from the archeal species *Thermococcus kodakaraensis* was found to have ATP-independent proteolytic activity when presented with unfolded proteins, but not those in their native conformations (Fukui *et al.*, 2002). Since the generally understood role of ATP in *E. coli* Lon is to aid in unfolding the protein structure before proteolysis, it may be that degradation of unfolded proteins could also occur in the absence of ATP, in a similar manner to its archaeal orthologue.

1.4 Aims and Hypothesis

The major aim of this project is to confirm that the Lon protease does play an important role in post-death metabolism by creating an *in vitro* model. This will be achieved by growing BW25113 wild-type strains of *E. coli* (WT) for various time points and comparing the growth to that of *Δlon*. As has been observed in previous studies, the WT strain is expected to be able to survive, as it has post-death metabolic activity. The *Δlon* strain is hypothesised to have an impaired post-death metabolism, meaning that its survival in the long-term stationary phase will be compromised.

It should be noted that death, within the context of this project, refers to a non-viable cell which cannot be seen when counting colony forming units (CFU) on an agar plate, as described in Finkel, 2006. An alternative description of death is often given as a cell which exhibits no metabolic activity (Mason, 1986). However, since the Lon produced by the dead cells is being hypothesised to metabolise the cell, this definition was felt to be overly simplistic, hard to measure, and may lead to confusion about when the cell stops being 'live'.

The secondary aim is, if a difference in post-death metabolism is observed, to investigate whether the *Δlon* mutation would be able to confer a GASP phenotype onto the population and if it would be able to outcompete the WT strain. This will be tested by growing WT/*Δlon* mixed cultures and observing the relative fitness of each strain at various time points.

2. Methods

2.1 Strains, Media, and Culture Conditions

2.1.1 Strains Used.

Table 1 illustrates the strains used throughout this project.

Table 1: Strains used throughout study.

Name	Antibiotic Resistance	Plasmid Promotor	Source
E. coli BW25113 (wild-type; WT)	-	-	Cann Lab Stocks
E. coli BW25113Δ*lon* (Δ*lon*)	Kam^R	-	Keio Collection (Baba *et al.*, 2006)
E. coli BW25113Δ*lon* :*p*BAD33-Lon (Δ*lon*R)	Cm^R Kam^R	araBAD	This Study
E. coli BW25113:*p*BAD33	Cm^R	araBAD	Cann Lab Stocks
E. coli BW25113Δ*lon* :*p*BAD33	Cm^R Kam^R	araBAD	This Study
E. coli BW25113: *p*ET-19b	Amp^R	T7	This Study
E. coli BW25113Δ*lon*: *p*ET-19b	Amp^R Kam^R	T7	This Study
E. coli BW25113 Δ*lon*:*p*ET24a-*Thermococcus onnurineus NA1* Lon (*Ton*Lon)	Kam^R	T7	(An *et al.*, 2010)

2.1.2 Media and Agar.

Unless otherwise specified, assume that all cells were grown in minimal media (M9). Cells not grown in M9, were grown in Luria-Bertani broth (LB; Miller's formulation; *Melford*).

M9 5x Salt solution (*Sigma Aldrich*) was made up as per manufacturer's instructions. For 250 mL of M9 media, 50 mL 5x salt solution was supplemented with 10 mL 50% (v/v) glycerol *(APC Pure),* 50 µL 1 M calcium chloride (CaCl$_2$; *Melford*) solution and 500 µL 1 M magnesium chloride (MgCl$_2$; *Melford*) solution. Care was taken to add the CaCl$_2$ before the MgCl$_2$ to prevent the salt precipitating out of solution. The supplemented salt solution was sterile filtered (0.22 µM pore size; *STARLAB*) into 190 mL sterile water and aliquoted into 50 mL aliquots.

LB and LB agar (*Melford*) were prepared as per the manufacturer's instructions. LB agar was stored at 60°C.

2.1.3 Antibiotics.

Ampicillin (*Melford*) and Kanamycin (*Melford*) were made up to concentrations of 100 mg/mL and 50 mg/mL respectively with MilliQ water before being sterile filtered (0.22 µM pore size; *STARLAB*). Chloramphenicol (*Melford*) was made up to 34 mg/mL with ethanol. All antibiotics were aliquoted into 1 mL Eppendorf's, stored at -20°C, and fully defrosted before use.

2.1.4 Selective Agar.

LB agar was prepared and stored as in 2.1.2. Agar was cooled until just starting to solidify and the antibiotic required was added to a final concentration of 0.05 mg/mL,

0.034 mg/mL or 0.1 mg/mL for Kanamycin, Chloramphenicol and Ampicillin respectively. Plates were poured and stored at 4°C.

2.1.5 Strain Storage.

Stocks were created for all strains through inoculating frozen master strains (-80°C) into LB with an appropriate antibiotic and incubating at 37°C with aeration (New *Brunswick Scientific Innova 40 Shaking Incubator;* 180 rpm) overnight. The cultures were prepared as 50% (v/v) glycerol stocks and stored at -80°C.

Agar plates for the strains were also created for quick access. 30 µL of an overnight culture was spread out using a sterile loop onto a petri dish containing approximately 20 mL of LB agar and any appropriate antibiotic. These plates were incubated overnight at 37°C before being wrapped in Parafilm and stored at 4°C.

2.1.6 Growth Conditions.

All strains used to inoculate an experiment were first grown up from glycerol stocks/plates overnight (approximately 18 hrs) at 37°C with continuous aeration (180 rpm) in either M9 or LB media. All cultures were always handled in a sterile environment.

2.2 Transformations

Multiple transformations were performed as part of this project to create the variety of strains seen above (2.1.1). Δ*lon* (a mutant of WT *E. coli* with the *lon* gene knocked out and replaced with a kanamycin resistance cassette) was transformed with pBAD33-Lon plasmid in order to create a rescue phenotype (Δ*lon*R). An empty pBAD33 plasmid was transformed into WT and Δ*lon* for control purposes. WT and

Δ*lon* were also transformed with an empty *p*ET-19b plasmid in order to confer ampicillin resistance onto the strains.

2.2.1 Plasmid Extraction.

Both plasmids had previously been transformed into *E. coli* DH5-α for long term storage at -80°C. The DH5-α strains were grown up overnight and the plasmids were extracted through use of a Monarch® plasmid miniprep kit according to kit instructions (*New England Biolabs*).

2.2.2 Creating Competent Cells.

Competent Δ*lon* and WT cells were made following the method from **(Chang *et al.*, 2017)**. A culture grown overnight (either WT or Δ*lon*; placed on ice for 20 mins prior) was centrifuged (Megafuge 40R; *Thermo Scientific*) at 4000 rpm and 4°C. Supernatant was discarded and pellet resuspended in 20 mL ice-cold 0.1 M $CaCl_2$ (*Melford*) before incubating for 30 mins on ice. The centrifuge step was repeated. Pellet was resuspended with 5 mL 0.1 M ice-cold $CaCl_2$ with 15% (v/v) glycerol *(APC Pure)* and aliquoted out for storage at -80°C.

2.2.3 Heat-shock transformation.

5 µL of plasmid was added to thawed competent cells and incubated for 30 mins on ice. Cells were then put in a 42°C water-bath for 30 s before placing in ice for 2 mins. 1 mL of LB was added to cells (pre-warmed) and cells were incubated for 1 hr at 37°C and shaken at 180 rpm. Cells were spread out on selective agar plates (Chloramphenicol or Ampicillin) and incubated overnight at 37°C. Colonies that grow

on selective agar plates had undergone transformation successfully. Colonies were picked and used for the creation of glycerol stocks (see 2.1.5).

2.3 Measuring Bacterial Growth

2.3.1 Colony Forming Units/mL (CFU/mL).

A cell culture was first serially diluted 1:10 (v/v) in sterile water before plating using the Miles-Misra method on to LB agar plates **(Miles, Misra and Irwin, 1938)**. 20 μL of diluted sample was added to a sectioned plate (1 section per serial dilution, max 8 sections). A sterile loop was used to spread out the sample to make for easier counting. For each culture, counts were performed in triplicate. Plates were incubated overnight at 37°C. The number of colonies were counted in sections with between 30 and 300 and used to determine the number of CFUs in 1 mL of original culture.

2.3.2 Optical Density (OD).

Unless otherwise specified, OD was measured using a hand-held spectrophotometer (*BioWave WPA CO8000*) at 600 nm. Readings were always made in triplicate.

2.3.3 Cell Viability Assay.

BacTiter-Glo™ Microbial Cell Viability Assay kit (*Promega*) was used as per manufacturer's instructions. BacTiter-Glo™ Reagent was made up, and 50 μL, along with 50 μL of sample, was added to a white sterile 96-well plate (*Greiner BIO-ONE*) in triplicate and mixed. A well of reagent mixed with media was also included as a background control. A variety of methods of mixing were trialled including an orbital shaker, shaking the plate by hand, and using a clean pipette tip to mix each well. The

pipette tip was the most effective. The plate was incubated at room temperature for 5 mins. Luminescence was recorded using a Synergy™ H4 plate reader *(BioTek)*.

2.3.4 Validation of OD.

A cell culture grown overnight was diluted with media in the following ratios: 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 (v/v). The OD of the samples was read, and CFU/mL determined. Validation was repeated using cells grown in both M9 and LB media. Pearson's correlation coefficient was calculated to determine if a correlation between the two tests exists.

2.3.5 Validation of Cell Viability Assay.

A cell culture grown overnight in M9 was diluted with M9 media in the following ratios: 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 (v/v). The OD of the samples was read, and BacTiter-Glo™ Microbial Cell Viability Assay kit performed (2.3.3). Validation was repeated using cells grown in and diluted with LB media. Any correlation between the amount of luminescence recorded and the OD was examined using Pearson's correlation test.

2.4 Methylene Blue

A working solution of 1.5% (w/v) methylene blue *(Sigma-Aldrich)* was made up. Methylene blue is an oxygen indicator which changes from blue to colourless in an oxygen poor environment. Cultures of WT *E. coli* were grown up overnight before OD was determined. Cultures were diluted to OD 0.05 with M9. 5 mL of diluted culture was added to two culture tubes (Pyrex® Culture Tube, with SVL® PBT screw caps; *DWK LIFE SCIENCES*) along with 50 µL of the working solution of methylene blue. One tube had its lid fully tightened (closed system) in order to prevent the exchange of

oxygen. The other had its lid loosely taped in place to allow for oxygen flow (open system) whilst still maintaining a sterile environment. A third tube had 5 mL of M9 alone added as a sterility control. Tubes were incubated with aeration at 37°C (*New Brunswick Scientific Innova 40 Shaking Incubator*). Tubes were observed and photographed every few days for 21 days to ensure sterility and the blue colour had remained. Concentrations and volumes of methylene blue used were based on the framework provided by **(Gibson, Harrison and Cox, 2021)**.

2.5 Measuring Bacterial Growth in the Log Phase

Cell cultures of WT, Δ*lon* and Δ*lon*R were grown up overnight in both LB and M9. 100 μL of each culture were knocked back to an OD of 0.05 and added to a sterile 96-well plate (*Falcon®*) with six technical replicates. The samples were incubated at 37°C in a shaking plate reader (SPECTROstar NANO; *BMG LABTECH*) for 1245 mins and OD readings at 600 nm were taken every 5 mins.

A one-way ANOVA was conducted on the endpoint OD values in order to compare the overall growth of the culture over the time frame. The data points taken from the first 250 mins were analysed to determine the K value, or rate of growth of the cultures, in the initial log phase. The K values were also subjected to a one-way ANOVA.

2.6 *In vitro* Model of Post-Death Metabolism

WT, Δ*lon* and Δ*lon*R cultures were grown up overnight in M9. The OD value of each culture was determined. The relevant volume of each culture was added to a culture tube (Pyrex® Culture Tube, with SVL® PBT screw caps; *DWK LIFE SCIENCES*) to reach

a final OD of 0.05, in a volume of 5 mL, using M9 as the dilutant. Each strain was set up in triplicate alongside a sterility control (5 mL of M9).

The 'day 0' OD and CFU/mL of the long-term growth assay cultures were measured, and the tubes were placed in a shaking incubator (N*ew Brunswick Scientific Innova 40 Shaking Incubator*; 37°C, 180 rpm) for 1, 5, 10, 15, 20 or 25 days. A final OD was then recorded, and the number of CFU/mL determined.

A two-way ANOVA with multiple comparisons (Tukey's test) was performed to compare the growth of each culture at each time point.

2.7 Determining the Protein Content of Supernatants

2.7.1 Separation of the Supernatant.

1 mL of culture was taken from 1, 5, 10, 15, 20 and 25 day long-term growth assays. The samples were centrifuged at 4000 rcf (Relative Centrifugal Force) for 10 mins (E*ppendorf Centrifuge 5424R*). The supernatant was removed carefully ensuring the cell pellet was not disturbed and put into sterile 1 mL Eppendorf's. Supernatants were stored at -80°C.

2.7.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gels.

SDS-PAGE gels were made comprising of two parts – a 5% stacking gel laid on top of a 12% running gel. For 15 mL of 12% running gel, 4.9 mL H_2O, 6.0 mL 30% (w/v) acrylamide mix *(BioRAD),* 3.8 mL 1.5 M Tris (*Melford*; pH 8.8), 0.15 mL 10% (w/v) SDS *(Melford),* 0.15 mL 10% (w/v) Ammonium persulfate (*Melford*) was mixed with 6 μL TEMED *(Sigma Aldrich),* ensuring that the TEMED is added last. This was poured into a clean gel casing and topped with a thin layer of isopropyl alcohol *(Sigma Aldrich)* in

order to maintain an even surface. The gel was left to set before the isopropyl alcohol was removed using clean blotting paper. The 5% stacking gel was mixed and poured on top of the running gel until it reached the top of the casing, and the comb was added. 10 mL of 5% stacking gel was made from 6.8 mL H_2O, 1.7 mL 30% (w/v) acrylamide mix, 1.25 mL 1 M Tris (pH 6.8), 0.1 mL 10% (w/v) SDS, 0.1 mL 10% (w/v) Ammonium persulfate and 1 µL TEMED. The gel was left to set before the comb was removed.

10-20 µL of sample was mixed with 4x SDS-PAGE loading buffer in a 4:1 ratio and loaded per well of the SDS-PAGE gel, alongside a Pre-stained Protein Ladder (*Thermofisher Scientific*). Gels were placed into the MiniPROTEAN Tetra System (*BioRAD*) and run in 1x SDS Running Buffer for 45-60 mins at 150 V.

Gels were placed in InstantBlue® Coomassie Protein Stain (*abcam*) and placed on a rocker for at least 15 mins. Gels were rinsed with MilliQ before being visualised by being placed on a bright light source and imaged with a phone camera.

2.7.3 Bicinchoninic Acid (BCA) Assays.

Protein standards were created using Bovine Serum Albumin (BSA; *fisher scientific*) at the following concentrations: 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/mL.

25 µL of standards and samples were added to a 96-well plate in triplicate along with 200 µL of BCA working reagent (Pierce™ BCA Protein Assay Kit, *ThermoFisher SCIENTIFIC*). The plate was incubated at 37°C for 30 mins before being cooled to room temperature. Absorbance was read at 562 nm in a plate reader (SPECTROstar NANO;

BMG LABTECH). Protein standards were used to create a standard curve from which the concentrations of the samples could be determined. A one-way ANOVA was carried out on the data along with Tukey's post-hoc test.

2.7.4 Pierce Assays.

Peptide standards were created using the Peptide Digest Assay Standard provided with the Pierce™ Quantitative Colorimetric Peptide Assay kit (*ThermoFisher SCIENTIFIC*). Standards were made to the following concentrations: 0, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 µg/mL.

20 µL of standards and samples were added to a 96-well plate in triplicate along with 180 µL of working reagent provided. The plate was incubated at room temperature for 30 mins. Absorbance was read at 480 nm in a plate reader (SPECTROstar NANO; *BMG LABTECH)*. Peptide standards were used to create a standard curve from which the concentrations of the samples could be determined. Data was analysed using a one-way ANOVA followed by Tukey's post-hoc test.

2.8 Casein Fluorescence

Supernatants (see 2.7.1 for preparation details) were fully thawed on ice along with M9 media, MilliQ water, and a solution of 2% glycerol (v/v; *APC Pure*). 60 µL of each sample was added to 40 µL of 5 mg/mL Fluorescein isothiocyanate (FITC) casein solution (*Sigma-Aldrich*) and vortexed. The mixture was incubated at 37°C and shaken for 1 hr at 200 rpm. During this incubation, the mixtures were covered in foil to prevent light-mediated FITC breakdown. 240 µL of Trichloracetic acid (50 mg/mL; *Sigma-Aldrich*) was added to each sample, vortexed and incubated at room

temperature for 2 hours. The samples were centrifuged at 10000 rcf for 5 mins before the supernatant was extracted leaving behind any excess FITC.

A HORIBA Flourolog-QM specrofluorometer was used to analyse the supernatant samples. Measurements of the emission spectra were taken between 475 nm and 600 nm. TRIS buffer was used to 'blank' the apparatus. Each sample was measured in triplicate. The data were normalised to the water 'blank' value which was set to 100 relative fluorescence units (RFU) and compared using a one-way ANOVA and tukey's post-hoc test for multiple comparisons.

2.9 Competition Assays

Methods in this section were adapted from Diggle *et al.,* 2007.

2.9.1 Competition Assays with a Static Ratio.

WT, Δ*lon* and Δ*lon*R cultures were grown up overnight in M9. The OD of these cultures was recorded, and relevant volumes added to M9 in culture tubes (Pyrex® Culture Tube, with SVL® PBT screw caps; *DWK LIFE SCIENCES*) to create the ratio of 97% WT to 3% Δ*lon* (or Δ*lon*R) and a final OD of 0.05. The exact starting ratio was determined by performing CFU/mL counts on both LB agar plates and selective LB agar plates containing kanamycin, to which Δ*lon* and Δ*lon*R are resistant, as described in *2.3.1*. Tubes were incubated at 37°C with continuous aeration (*New Brunswick Scientific Innova 40 Shaking Incubator*), for various time periods - specifically: 250 mins, 1200 mins, 5 days, 11 days, 21 days and 22 days. The final ratio after incubation time was determined in the same manner and the change in percentage composition of the population was determined. Data was analysed through a two-way ANOVA followed by Šidák's multiple comparisons test.

2.9.2 Competition Assays where the Start Ratio is Varied.

WT and Δ*lon* cultures were grown up overnight in M9. The OD of these cultures was recorded, and relevant volumes added to M9 in PYREX® culture tubes to create the desired ratios. These ratios were 99.9:0.1, 99:1, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90 WT:Δ*lon*. The exact starting ratios were determined by performing CFU/mL counts on both LB agar plates and selective LB agar plates containing kanamycin, to which Δ*lon* is resistant, as described in *2.3.1*. Tubes were incubated at 37°C for 11 days in a shaking incubator. The final ratio after incubation time was determined in the same manner and the change in percentage composition of the population was determined.

The varied competition assay was repeated with a five day incubation time. Here only the desired starting ratios of 99.9:0.1, 99:1, 95:5, 90:10, and 70:30 WT:Δ*lon* were used.

The relative fitness of the Δ*lon* mutant (*w*) was calculated through use of the following formula which compares the rate of growth of Δ*lon* to that of the WT population.

$$w = (x_2(1 - x_1))/(x_1(1 - x_2))$$

Here, x_1 is the initial proportion of the mutant in the population and x_2 is the final proportion. Data was fitted with a simple linear regression and R^2 value calculated.

2.10 Expression and Purification of TonLon

The expression of Lon from *T. onnurineus* was performed according to the method developed by **(An *et al.*, 2010)**. *E. coli* BW25113Δ*lon* was transformed with a *p*ET24a

plasmid containing the *lon* gene from *T. onnurineus.* The gene product will hereafter be referred to as *Ton*Lon.

The novel *E. coli* strain was grown in LB media supplemented with 50 μg/mL Kanamycin at 37°C until it reached an OD of 0.5. *Ton*Lon expression was induced by the addition of 750 μL 1 M isopropyl β-d-1-thiogalactopyranoside *(Melford)* and the temperature was dropped to 18°C. Cells were harvested after 18 hrs and resuspended in 50 mM Tris–HCl pH 8.0 (*Melford*) and 500 mM KCl (*Melford*).

Cells were centrifuged at 4000 rpm for 20 mins and the supernatant was poured off. The remaining cell pellets were sonicated to produce the cell lysate. The lysate was centrifuged at 20,000 g for 60 mins at 4°C to separate the insoluble fraction out. The supernatant was run down a nickel-nitrilotriacetic acid column (*Cytiva*) and washed with the above buffer supplemented by 10 mM imidazole (*Fisher Scientific*)*.* A 50 mM Tris–HCl pH 8.0, 500 mM KCl, 300 mM imidazole buffer was used to elute the *Ton*Lon fraction before it was concentrated. The concentrated sample was loaded onto a Superdex 300 HR 16/600 column (*Cytiva*). The column had previously been equilibrated with a 50 mM Tris-HCL pH 8.0, 15 mM $MgCl_2$ *(Melford)* buffer. *Ton*Lon eluted after approximately 60 mL (using a flow rate of 1.5 mL/min). It was dialysed with a buffer containing 50 mM Tris–HCl pH 8.0 and 0.4 mM $MgCl_2$and concentrated to a final concentration of 30 mg/mL. Protein was stored in 1 mg/mL and 100 μg/mL aliquots.

2.11 Statistics and Figure creation

Statistical analysis was performed using GraphPad Prism (GraphPad Prism version 8.3.1 for Windows, *GraphPad Software*). Results were considered significant if the

relevant statistical test yielded a p value of < 0.05. All experimental results have been performed at a minimum in triplicate with three independent biological repeats. Where shown on figures, error bars represent the standard error of the mean unless specified. Where statistical significance has been indicated on a figure, a standard star system was used: * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, and **** indicates $p \leq 0.0001$.

3. Results

3.1 Calibration and Validation

3.1.1 Optical Density can be used as a valid method of measuring bacterial growth.

The growth of *E. coli* can be measured in many ways, with two of the most common being reading the optical density (OD) of the culture at 600 nm and counting the number of colony-forming units per mL of culture (CFU/mL counts). OD is measured by determining the amount of light that can pass through a culture and using that as an estimate of how many cells are. CFU/mL counts are found by serially diluting a culture, spreading each dilution out on a petri dish, and counting the number of resultant colonies on the plate to find how many cells or colony-forming units are present per mL of the original culture.

Calibration curves, using *E. coli* grown in either M9 or LB media, as seen in figure 5, ensured that OD could be used to successfully approximate the number of CFU/mL simply and quickly (see supplementary figures 1 and 2 for all replicates). It is worth noting, however, that this relationship was only investigated for cultures in the late log and early stationary phase. It is unlikely to hold during the death phase, where a high proportion of the culture dies off, leading the OD reading to become inflated by the extracellular products in the culture which are not living cells.
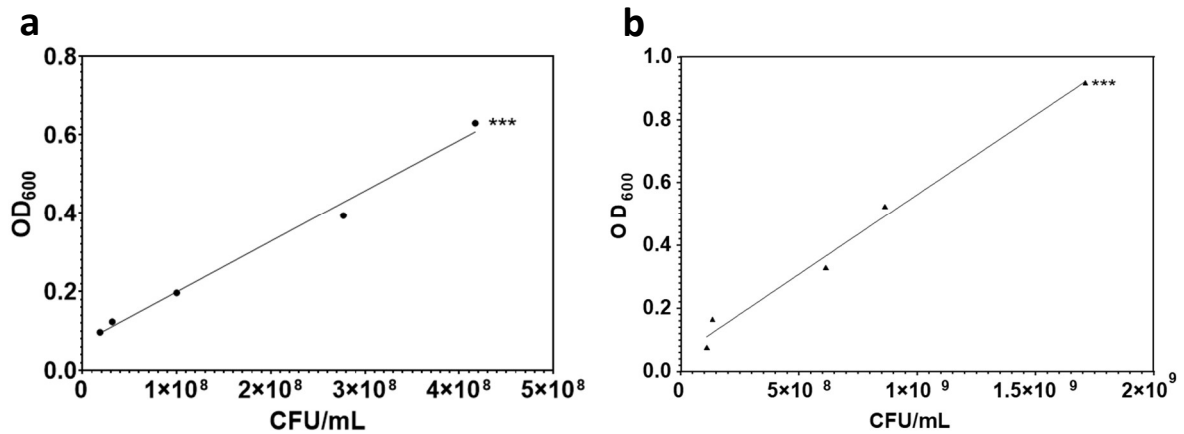
Figure 5: Standardization Curves showing how the OD of a culture grown in M9 (a) or LB (b) depends on the number of CFU/mL present. Experimental points denote the average of three technical replicates with error bars representing the standard error of the mean (SEM). R^2 values were calculated through a simple linear regression (**a**: $R^2 = 0.9910$, **b**: $R^2 = 0.9983$). Correlation is statistically significant and calculated through Pearson's correlation coefficient (**a**: ($r(3) = 0.9955$, $p = 0.0004$), **b**: ($r(3) = 0.9941$, $p = 0.0005$)).

3.1.2 Cell Viability Assays are a valid method of measuring bacterial growth.

A third method of measuring bacterial growth is commercially available kits such as the BacTiter-Glo™ Microbial Cell Viability Assay kit (*Promega*), which uses a reaction between luciferin and ATP which causes light to be emitted. The amount of light produced is proportional to the amount of ATP in the sample and, therefore, the number of cells present.

Figure 6 demonstrates how the amount of luminescence produced by a sample is proportional to the OD of the sample. However, this kit was soon abandoned as it failed to give consistent results, and even small variances in room temperature or the amount of mixing would produce significant changes in the luminescence recorded. Furthermore, a third method of measuring *E. coli* growth was deemed unnecessary.
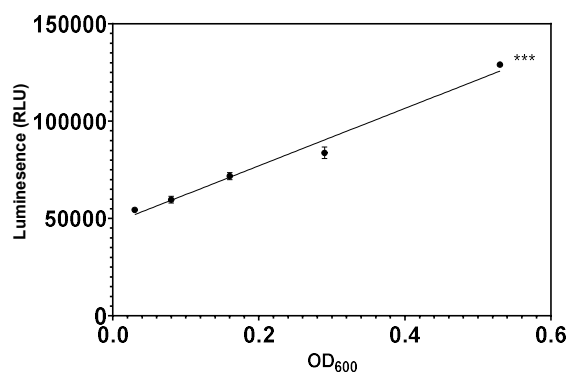
Figure 6: Luminescence given off by BacTiter-Glo™ Microbial Cell Viability Assay kit (*Promega*) is dependent on the OD of the sample. Experimental points denote the average of three technical replicates with error bars representing S.E.M. R^2 values were calculated through a simple linear regression ($R^2 = 0.9827$) Correlation is statistically significant and calculated through Pearson's correlation coefficient ($r(3) = 0.9913$, $p = 0.0010$). A repeat is shown in supplementary figure 3.

3.1.3 E. coli cultures do not enter anaerobic conditions during 25 days of continuous growth.

This study aimed to examine the effects of nutrient stress on the post-death metabolism of *E. coli,* so any potential stress caused by a low-oxygen environment had to be ruled out.

E. coli are facultative anaerobes meaning that they prefer an oxygen-rich environment although this is not a requirement **(Unden *et al.*, 1994)**. Methylene blue is an oxygen indicator which becomes colourless in a low-oxygen environment. Cultures of WT *E. coli* were grown in the presence of the indicator for 25 days either in a sealed culture tube (closed system) or in a culture tube where the lid was loosely taped in place (open system where oxygen could be easily replenished; figure 7). Whilst there was a slight colour change in the closed system after seven days as the blue colour became slightly less intense, the colouring remained strong throughout all 25 days (see supplementary figure 4). No difference was observed in the colouring of the open system at any time although some culture volume was lost, presumably

due to evaporation. Sterility controls, not pictured, showed these losses did not cause any issues with contamination. Ideally, the concentration of the blue colour would have been measured using a colorimeter each day in order to get quantitative results, but time constraints did not allow for a second repeat. Furthermore, it would be preferable to have an anaerobic control to demonstrate the maximum colour change when oxygen is not present, but it was not possible to create such an environment with available equipment. A mass of literature is available, however, showing that this test is both sensitive and reliable with the methylene blue fully becoming colourless at around 0.1% oxygen (Sumitani *et al.*, 2004; Duan, Fang and Wang, 2021; Gibson, Harrison and Cox, 2021).

Despite these limitations, the experiment does show, however, that within the time scales required for this study, sealed culture tubes do not create a low oxygen environment and no additional stress is conferred onto the bacterial cultures.
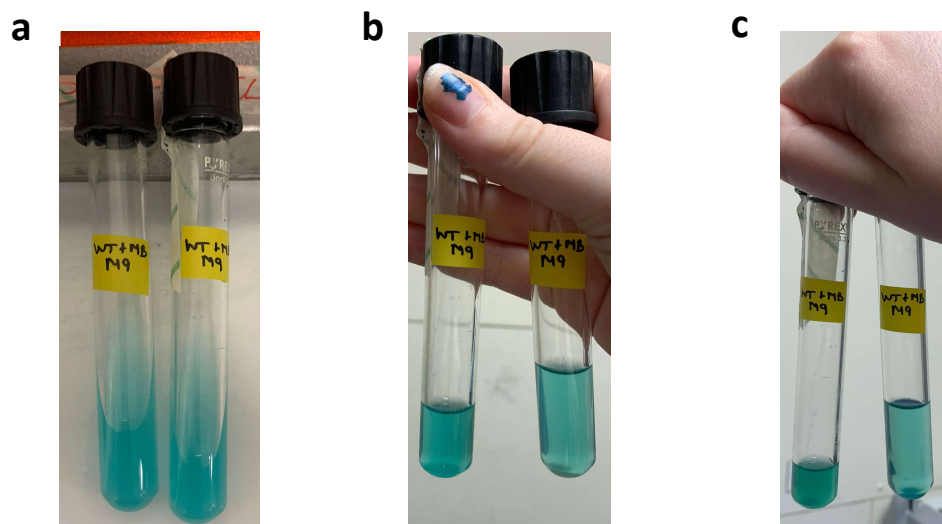
Figure 7: Appearance of WT cultures with the addition of Methylene Blue. The tubes were imaged at the following time points: Day 0 (**a**; LHS closed system, RHS open system), Day 7 (**b**; LHS open system, RHS closed system), and Day 21 (**c**; LHS open system, RHS closed system). The blue colour became less intense in the closed system by day 7 however remained present throughout. The volume dropped in the open system due to evaporation losses.

3.1.4 The Δlon strain produces no detectable amount of Lon whilst the ΔlonR and WT strains produce comparable amounts.

This study used various knock-out and rescue strains of *E. coli* BW25113. The *Δlon* knock-out strain was taken from the Keio collection. A rescue phenotype was created by inserting a pBAD33-Lon plasmid into the *Δlon* knock-out. The resultant strain is referred to as *Δlon*R. The *Δlon* was confirmed through western blot not to produce any Lon, whilst the *Δlon*R strain produces comparable amounts of Lon to the WT strains (figure 8). This work was performed by S.E.R Gibson and included here for completeness.
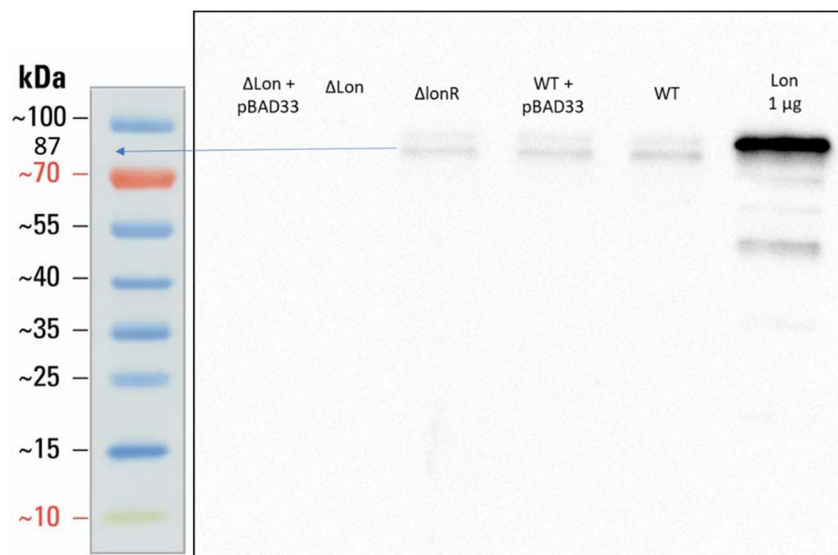
Figure 8: Western Blot Analysis of Lon produced by various strains of BW25113 *E. coli.* *Δlon* and *Δlon*+pBAD33 strains do not produce any Lon protein. *Δlon*R, WT and WT+pBAD33 strains all produce a near identical amount of Lon. Figure modified and used with permission from S.E.R Gibson.

3.2 Investigating Differences in Growth During Log Phase

3.2.1 Δlon grows slower during the exponential log phase than WT.

WT, *Δlon,* and *Δlon*R cultures were grown in M9 and LB media for 1245 mins (20 hrs), where OD was recorded at 5 min intervals. The first 250 mins of this growth

constitute the exponential log phase in both media. This initial growth rate (K) was calculated using GraphPad Prism (figure 9).

As expected, the growth rate for all strains was approximately 10x higher in LB than in M9, as nutrients are plentiful and usable. This differs from minimal media where all building blocks, such as amino acids and nucleotides, must first be synthesised before division and growth can occur. More surprisingly, however, the Δlon strain grew at a rate that was significantly slower during early log phase than the WT or the rescue strains. There was no difference between the growth of the WT and rescue strain. The slowing effect on the Δlon strain's growth was true regardless of whether nutrients were readily available. This indicates that this effect is unrelated to nutrient stress and, therefore, to nutrient recycling.
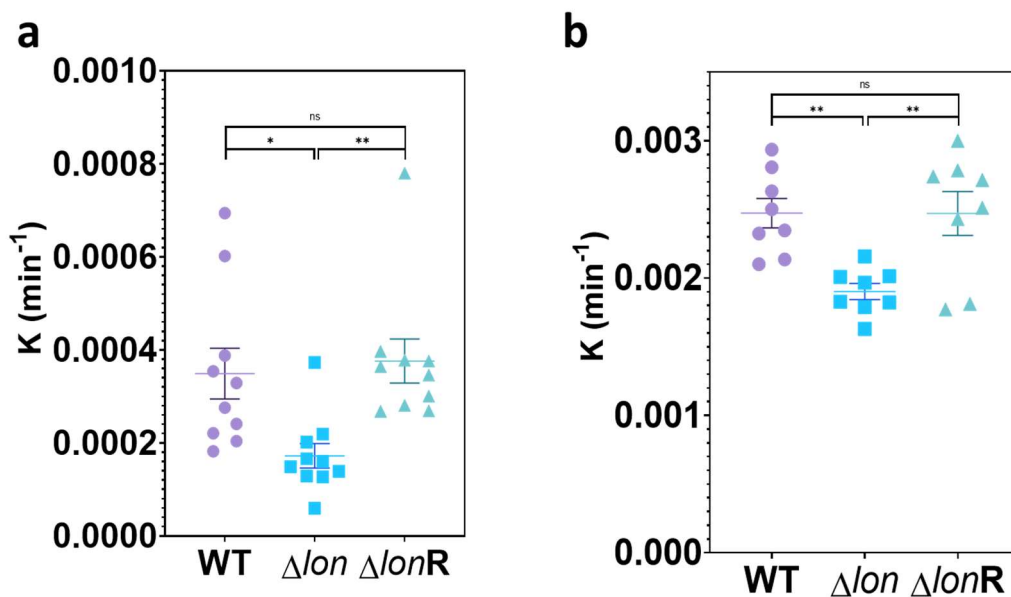
Figure 9: Δlon has a significantly slower growth rate than WT when grown in M9 (a) and LB (b). Strains were grown in the respective media for 1245 mins in a shaking plate reader with OD being measured at 5 min intervals. The first 250 mins of growth constituted the exponential log phase and was used to calculate the K value. Data (**a**: n = 10, **b**: n = 8) is analyzed with a one-way ANOVA (**a**: $F_{(2, 27)}$ = 6.216, p = 0.0060 **b**: $F_{(2, 21)}$ = 8.016, p = 0.0026). Tukeys multiple comparison test revealed that in both the M9 and LB conditions, WT and ΔlonR grew at a rate that was significantly faster than the Δlon strain but there was no significant difference between the growth of WT and ΔlonR. P values were all <0.03. Error bars depict S.E.M.

3.2.2 Δlon has a growth advantage over WT after 1245 mins.

WT, *Δlon,* and *Δlon*R cultures were grown in M9 or LB for 1245 mins (20 hrs) in a shaking plate reader from a starting OD of 0.05. OD was recorded at the start and end of the growth period, and the total change over the period was calculated. A one-way ANOVA was used to compare the final change in OD values, followed by Tukey's test for multiple comparisons.

When cultures were grown in LB (figure 10b,d), there was no difference in the total growth that occurred over the 20 hrs. Strikingly, however, when grown in minimal media, *Δlon* had a significantly higher total change in OD than either the WT or *Δlon*R (figure 10a). This confirms that the Lon protease is active when under nutrient-stress conditions but the presence of Lon causes a reduction in growth. The growth-dampening effect of Lon comes into effect as the WT strain moves into the stationary phase of growth, whereas the *Δlon* strain appears to continue in the log phase (figure 10c).

The difference in growth of the WT and *Δlon*R strains was also significant. This is likely an indication that the *Δlon*R strain is only a partial rescue of the mutant, causing it to behave neither like the WT nor the *Δlon* strains.
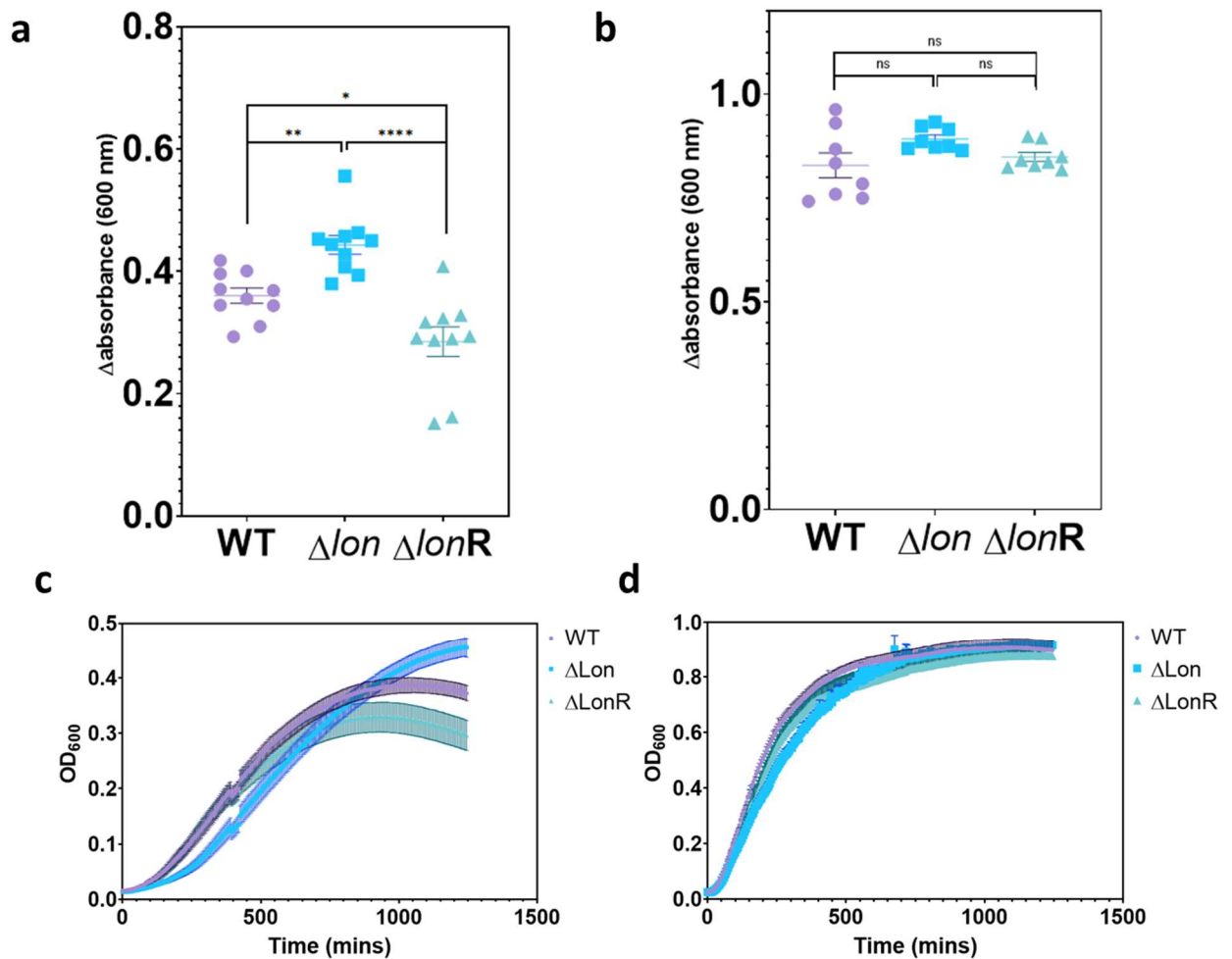
Figure 10: Δ*lon* has a significant growth advantage over WT when grown in M9 (a, c) but not in LB (b, d). Strains were grown in the respective media for 1245 mins in a shaking plate reader with OD being measured at 5 min intervals. Both the complete growth curves (**c**, **d**) and total change in OD are shown (**a**, **b**). Data (**a:** n = 10, **b**: n = 8) is analyzed with a one-way ANOVA (**a**: $F_{(2, 27)} = 19.18$, $p < 0.0001$ **b**: $F_{(2, 21)} = 2.851$, $p = 0.0803$). This was followed with Tukey's multiple comparisons test for growth in M9 where all strain's growth differed significantly with p always < 0.02. Error bars depict S.E.M.

3.3 *In vitro* model of Post-death Metabolism

The experiments reported in 1.2 were performed using lysates stressed by the growth process. These stressed lysates were added to unstressed WT *E. coli,* and any growth boost conferred by the lysates was observed. This experiment does not accurately reflect how bacteria are commonly found in nature. Here, it is more likely that the dead and living *E. coli* would be under the same stress and nutrient deprivation.

Therefore, this project aimed to create an *in vitro* model of this post-death metabolism.

The original methodology employed involved growing cultures as described in 2.6. Instead of varying how long the cultures were grown for and measuring the CFU/mL at the start and end of every experiment, cell viability assays were performed daily and CFU/mL was recorded every few days. This method, however, led to the cultures consistently becoming contaminated before the goal of 25 days could be reached (data not shown). Efforts were made to overcome this by using OD measurements alone to record the progress and by using strains of *Δlon* and WT which had ampicillin resistance conferred onto them. The OD readings alone were not effective at showing any changes to the cultures after around five days (figure 11). They did suggest, however, that the WT cultures followed the standard growth pattern of *E. coli,* whereas the *Δlon* strain did not appear to enter a death phase, instead having an extended stationary phase.
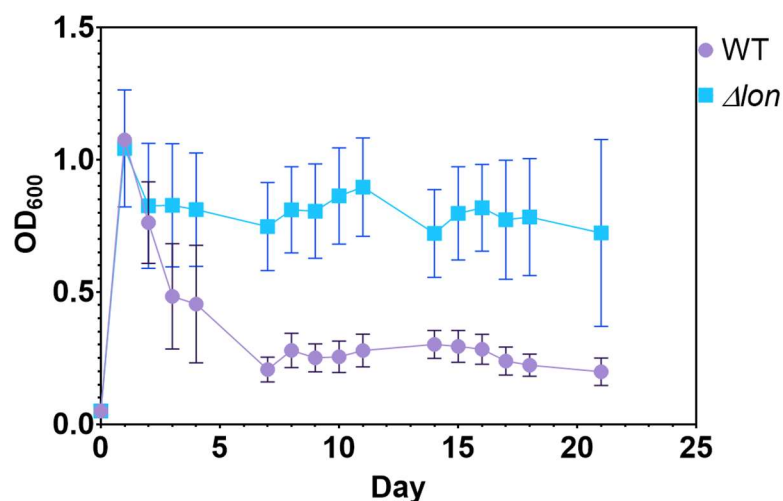
Figure 11: After 21 days of growth, the Δ*lon*+amp plasmid (blue square) strain does not appear to enter a true death phase as seen in the WT+amp plasmid (purple circle) strain. OD readings were made once a day using a handheld OD meter. For all data, n = 4. Error bars represent S.E.M.

After observing the above, the long-term growth assay methodology described in 2.6 was used. The non-ampicillin resistant strains were grown in M9 for various times, and both OD and the number of CFU/mL were recorded on Day 0 and at the last time point. The final difference between methodologies was that, instead of plating 10 μL of each dilution on an agar plate and counting segments with 3-30 colonies as previously done, 20 μL was now plated and sections with 30-300 colonies counted.

Figure 12 shows an overview of the data produced by this revised methodology. The OD data does not show the clear separation between the WT and *Δlon* strains previously seen. As the separation is still seen in the CFU/mL data, however, this is likely since the data shown in figure 11 comprises four replicates all set up from the same original overnight stock and so has less variation. All replicates (between 6 and 12 for each data point) shown in figure 12 were completely independent, leading to more variation, and a less distinct separation.
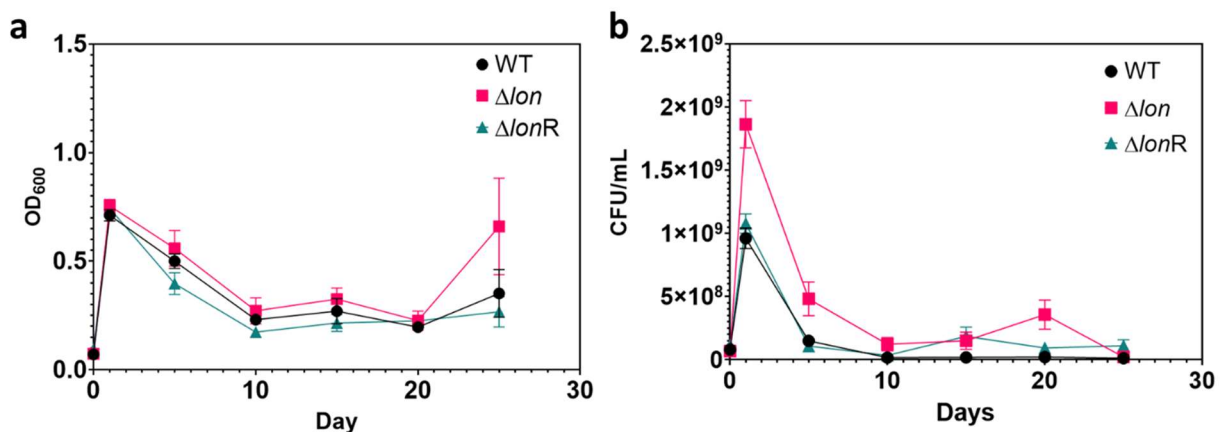
Figure 12: An overview of the growth and death of WT (black circle), *Δlon* (pink square) and *Δlon*R (green triangle) strains over 25 days using OD (a) and CFU/mL (b) to measure. Cultures were grown in M9 for 1, 5, 10, 15, 20 or 25 days from a start OD of 0.05. OD and CFU/mL were recorded on the 1st and last day of growth. OD readings were made using a handheld OD meter. CFU/mL was recorded through plating serial dilutions of cultures onto solid LB agar and counting colonies produced. Every point represents the mean of at least 6 independent biological replicates. Error bars represent S.E.M.

Figure 13 presents the CFU/mL data split up by day. All data was analysed by two-way ANOVA and then by Tukey's multiple comparison test (see supplementary table 1 for full details). As seen in 3.2, after one day, the *Δlon* strain has a growth advantage over the WT and *Δlon*R strains. All strains exhibit a significant decrease in number between days one and five to approximately a quarter of the day one level as they enter a death phase. However, the timing and extent of these phases differs massively. Between one day and five days of growth, the number of bacteria present in the WT culture decreased six-fold. This death phase continues until at least day 10. WT bacteria then enter the fifth and final stage of bacterial growth – the long-term stationary phase. The population fluctuates slightly but stays consistently similar or lower than the day 0 values.

Until day 15, the *Δlon* population stays at a significantly higher viable cell count than the WT, as its death phase was not as severe. After 15 days of growth and death, the *Δlon* strain is no longer significant to the WT but still retains the higher population. The population of *Δlon* increased by a factor of 2.4 over the next five days, whereas the number in the WT strain remained approximately the same. However, after the full 25 days, the *Δlon* has died off to such a level that it is now at a lower (although statistically insignificantly) level than the WT strain. Some individual replicates of the *Δlon* strain at this time point showed no viable colonies remaining.

The rescue strain behaves identically to the WT strain until day 10. Between day 10 and day 15, however, the *Δlon*R strain also boosts its growth, now meaning that there are significantly more bacteria in the *Δlon*R culture than in the WT cultures at the same point. The number of *E. coli* in the *Δlon*R culture stays consistent from then on.

However, slight fluctuations between the exact number present in the WT and *Δlon*R

cultures lead to changes in whether the difference is significant or not.
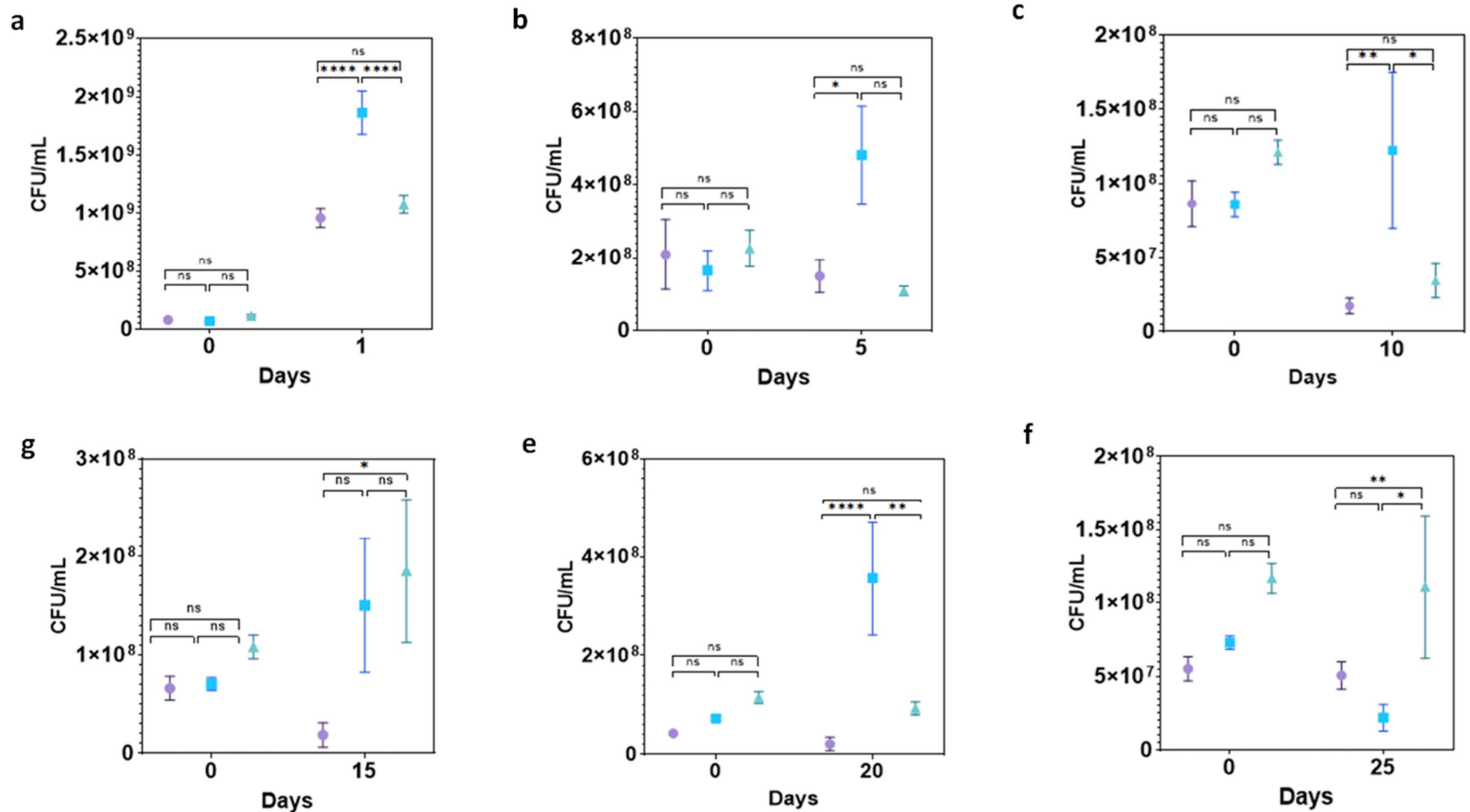
Figure 13: Δ*lon* (blue square) cultures grow to a higher number of CFU/mL than either the WT (purple circle) or Δ*lon*R (green triangle) and retains this growth advantage until 25 days. Cultures were grown in M9 for 1 (**a**), 5 (**b**), 10 (**c**), 15 (**d**), 20 (**e**) or 25 (**f**) days from a start OD of 0.05. OD and CFU/mL were recorded on the 1st and last day of growth. OD readings were made using a handheld OD meter. CFU/mL was recorded through plating serial dilutions of cultures onto solid LB agar and counting colonies produced. Every point represents the mean of at least 6 independent biological replicates. Error bars show S.E.M.

3.4 Investigating the Protein Makeup of Extracellular Environments at Different Phases of the Growth Cycle

Having established an *in vitro* long-term growth model to demonstrate post-death metabolism, the next step was to examine if there were any corresponding differences in the extracellular environment. To achieve this, BCA assays, Pierce Assays and SDS-PAGE gels were run to determine and visualise the protein content released into the bacteria's extracellular environment. The strains were grown for 1, 5, 10, 15, 20, or 25 days, and cells pelleted, leaving only the extracellular material in the supernatants.

3.4.1 SDS-PAGE Gel Electrophoresis does not show a difference in protein content of the extracellular environment.

The raw supernatants from 1, 5, 10, and 15 days were run down an SDS-PAGE gel with the hope that any difference in the total protein makeup would be visible.

Only a very faint trace of protein could be observed in any supernatant and no protein bands of interest could be identified, as can be seen in figure 14.

3.4.2 There is no difference in supernatant protein content between strains after the log growth phase.

The BCA assay is a standard assay used to determine the total protein content in a solution. Peptides larger than two amino acids long and containing tyrosine, tryptophan, or cysteine react with Cu^{2+} ions reducing them into Cu^+ ions – this is known as the Biuret reaction. BCA then reacts with the Cu^{2+} in a 2:1 ratio, creating a

metal complex with a deep purple colour that strongly absorbs light at 562 nm (Wiechelman, Braun and Fitzpatrick, 1988).
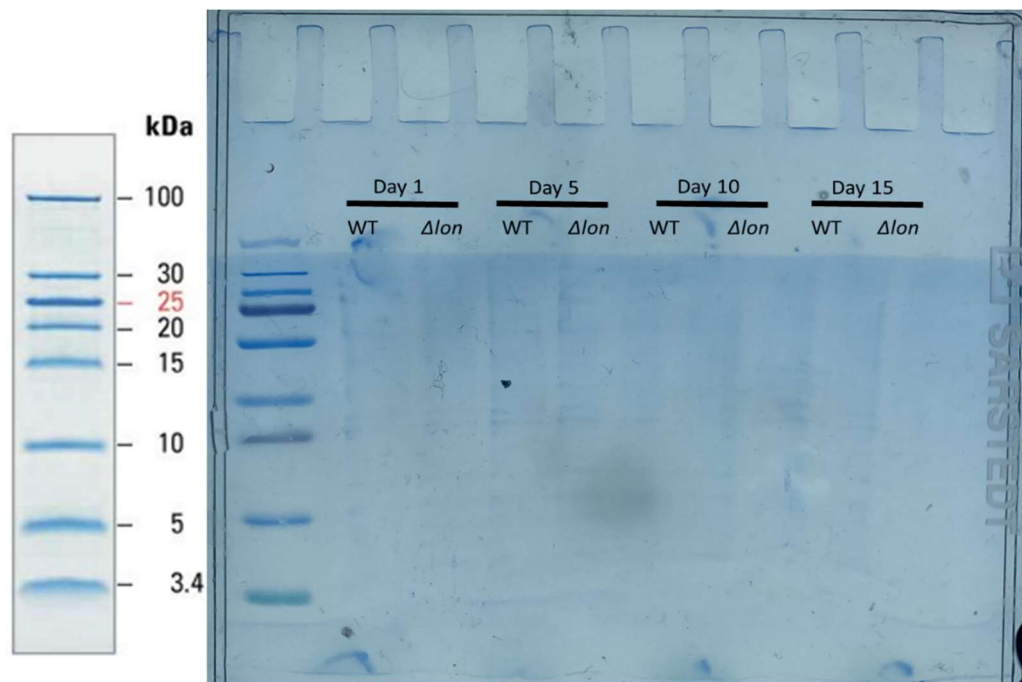
Figure 14: SDS-PAGE analysis of supernatants taken from WT and *Δlon* cultures grown for 1, 5, 10 or 15 days.

The concentration of protein in the supernatants (figure 15a) produced from the long-term growth assays increases from day one to day five, which is to be expected as the bacteria run out of nutrients and start to die off, releasing protein into the supernatant. After day five, however, all supernatants contain similar protein levels. The protein concentration in the WT supernatant decreases by a factor of 1.6 between day five and day 25. However, this change is not significant when investigated with a one-way ANOVA ($F_{(17, 35)}$ = [3.155], p = 0.0020) with Tukey's post hoc test (p = [0.3326], 95% C.I. = [-93.68, 675.9]). There is no similar trend when looking at the *Δlon* or *Δlon*R strains.

3.4.3 There are fewer peptides present in the WT extracellular environment after the log phase than the Δlon's.

The Pierce™ Quantitative Colorimetric Peptide Assay kit uses a modified BCA reaction as described above. Instead of BCA being used to chelate the Cu^+ ion produced by the biuret reaction, an alternative molecule is used, allowing the assay to be specific for short-chain peptides. ThermoFisher has not released any additional information about how this proprietary chemical may alter the BCA reaction in order to be this specific.

The results of the Pierce assay are presented in figure 15b. The data was analysed through a one-way ANOVA ($F_{(17, 35)}$ = [6.017], P<0.0001). The essential summary
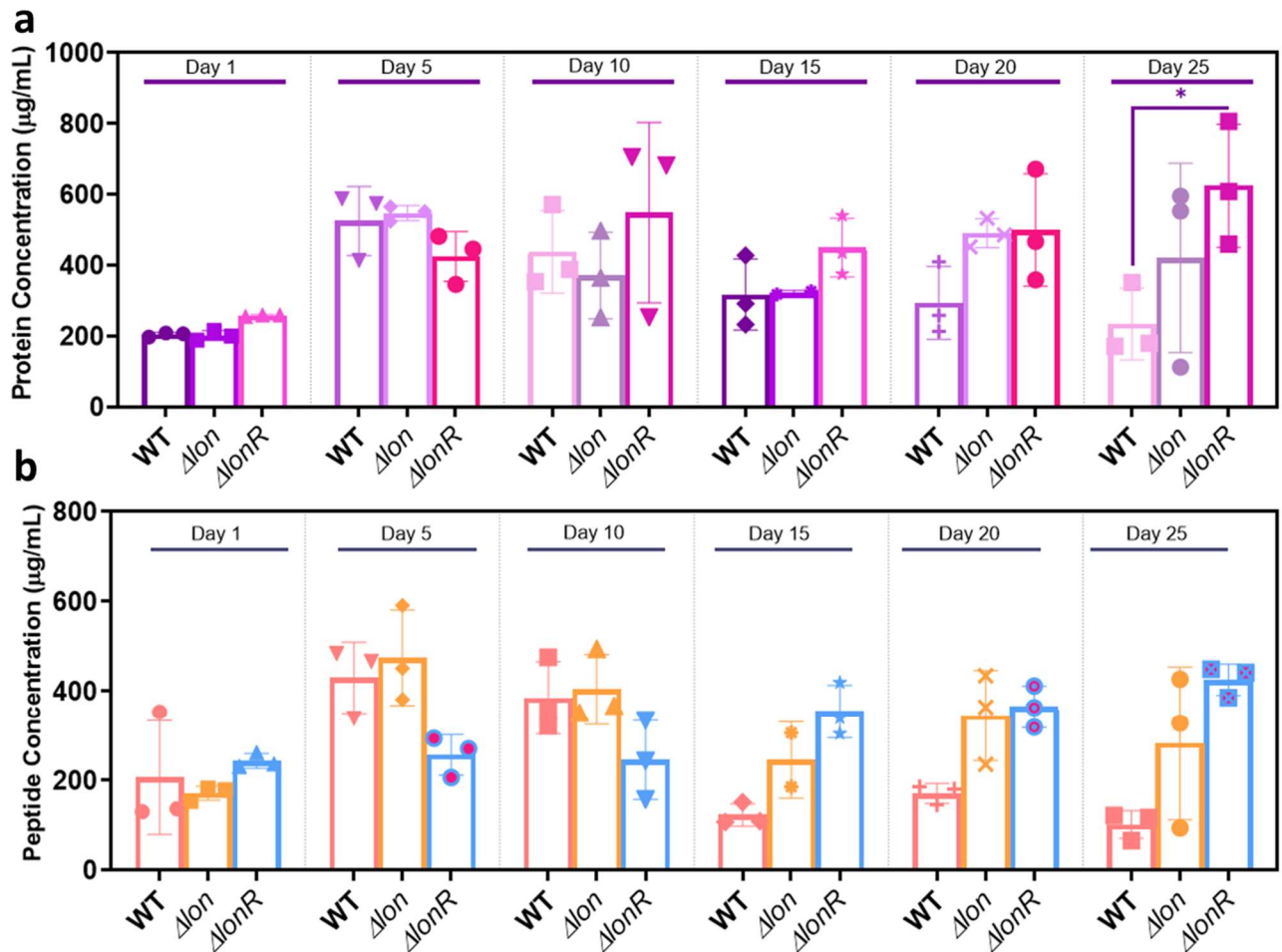
Figure 15: Protein concentration (a) remains constant across all supernatants, but peptide concentration (b) decreases in the WT from day five. a) Protein concentration was determined through use of the BCA assay. One-way ANOVA with Tukey's post-hoc test was used to compare all data (N = 3). Error bars represent Standard Deviation (SD). **b)** Peptide concentration was calculated using the Pierce™ Quantitative Colourmetric Peptide Assay kit. Error bars represent SD. N = 3 for all data. Statistical significance (one-way ANOVA followed by a Tukey test) is shown in Table 2.

statistics for Tukey's multiple comparisons test are displayed in Table 2. The full statistical output is given in supplementary table 2.

Table 2: Summarised results of Tukey's multiple comparisons test comparing the concentrations of peptides in the supernatants of the WT, *Δlon* and *Δlon*R strains collected after 1, 5, 10, 15, 20 and 25 days of growth.

Comparison	Mean Difference	95% Confidence Interval of Difference	Significant	P Value
LGA-1 WT vs. LGA-5 WT	-223.1	-465.0 to 18.82	ns	0.0985
LGA-1 *Δlon* vs. LGA-1 *Δlon*R	-72.00	-313.9 to 169.9	ns	0.9994
LGA-1 *Δlon* vs. LGA-5 *Δlon*	-303.2	-545.1 to -61.26	**	0.0041
LGA-1 *Δlon*R vs. LGA-5 *Δlon*R	-13.56	-255.5 to 228.4	ns	>0.9999
LGA-1 *Δlon*R vs. LGA-10 *Δlon*R	-2.518	-244.4 to 239.4	ns	>0.9999
LGA-5 WT vs. LGA-10 WT	44.89	-197.0 to 286.8	ns	>0.9999
LGA-5 WT vs. LGA-15 WT	307.1	65.18 to 549.0	**	0.0034
LGA-5 WT vs. LGA-20 WT	259.3	17.33 to 501.2	*	0.0256
LGA-5 WT vs. LGA-25 WT	328.4	86.44 to 570.3	**	0.0013
LGA-5 *Δlon* vs. LGA-10 *Δlon*	70.96	-171.0 to 312.9	ns	0.9995
LGA-5 *Δlon*R vs. LGA-10 *Δlon*R	11.04	-230.9 to 253.0	ns	>0.9999
LGA-10 WT vs. LGA-15 WT	262.2	20.30 to 504.1	*	0.0227
LGA-10 WT vs. LGA-20 WT	214.4	-27.56 to 456.3	ns	0.1323
LGA-10 WT vs. LGA-25 WT	283.5	41.55 to 525.4	**	0.0095
LGA-10 *Δlon* vs. LGA-15 *Δlon*	157.8	-112.7 to 428.3	ns	0.7457
LGA-10 *Δlon*R vs. LGA-15 *Δlon*R	-108.5	-350.4 to 133.4	ns	0.9570
LGA-15 WT vs. LGA-20 WT	-47.85	-289.8 to 194.1	ns	>0.9999
LGA-15 *Δlon* vs. LGA-20 *Δlon*	-99.11	-369.6 to 171.4	ns	0.9936
LGA-15 *Δlon*R vs. LGA-20 *Δlon*R	-10.15	-252.1 to 231.8	ns	>0.9999
LGA-20 WT vs. LGA-25 WT	69.11	-172.8 to 311.0	ns	0.9996
LGA-20 *Δlon* vs. LGA-25 *Δlon*	61.85	-180.1 to 303.8	ns	>0.9999
LGA-20 *Δlon*R vs. LGA-25 *Δlon*R	-60.74	-302.7 to 181.2	ns	>0.9999

As with the BCA assay, there was an increase in the number of peptides present in the supernatant between the one-day and five-day samples for both the WT and *Δlon* strains, although this change was only significant for *Δlon.* After this initial increase, however, the *Δlon* peptide concentration remains constant for the next 20 days. This

contrasts with the WT peptide concentrations, which remain at a similar level until day 10 but decrease significantly by day 15. This new low level of peptides remains stable for the remainder of the time. No difference in the concentration of peptides in the *Δlon*R supernatants was observed.

3.5 There are differences in the protease activity in the WT extracellular environment compared to that of the Δlon

After the observation that the WT extracellular environment contains fewer peptides after five days of growth than that of the *Δlon's*, an analysis of the supernatants was performed. This was to confirm that this difference was due to the lack of the extracellular Lon protease. The general protease activity of the supernatants taken from the *Δlon* strain at different time points was hypothesised to be lower than those of the WT strains.

The casein fluorescence assay uses a Fluorescein isothiocyanate (FITC) tagged casein protein (Twining, 1984). The casein and FITC bond can be cleaved by a protease, releasing the tag. The resulting fluorescence can be read at 520 nm and indicates the amount of proteolysis that a sample can achieve. Whilst the original paper only described this cleavage in response to trypsin, chymotrypsin, subtilisin and elastase, the Cann lab has demonstrated previously Lon's ability to also digest the linker (Twining, 1984).

Figure 16 shows the results of this analysis. As glycerol is known to fluoresce and to affect the results of any fluorescence data, a 2% glycerol sample – the same final concentration as M9 media - was included as a control (Feldman *et al.*, 2021). This

sample has approximately double the fluorescence of the M9 media control, an effect possibly explained by the buffering abilities of the salts within the media.

Surprisingly, there was no difference between the protease activity of any of the supernatants, either when compared between themselves or when compared to the
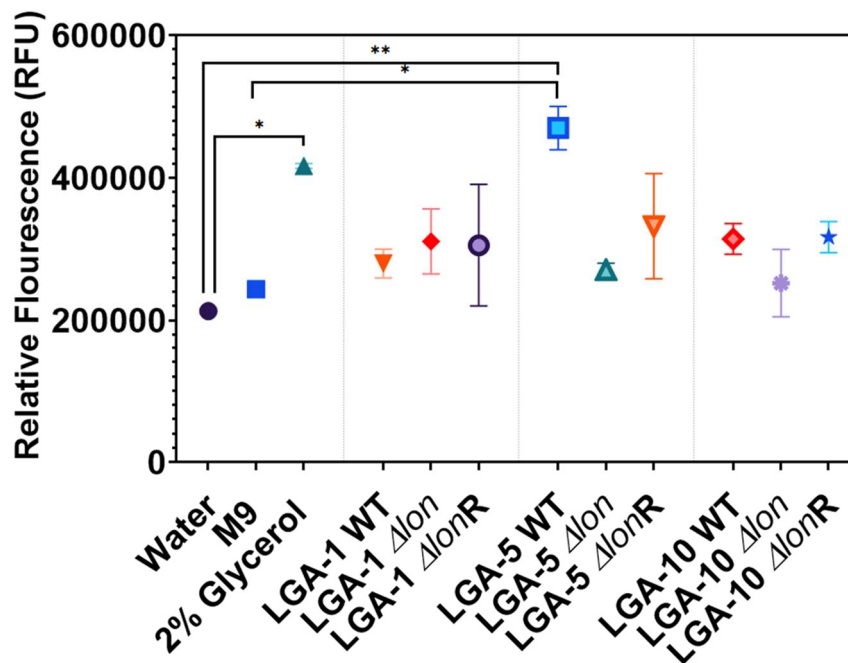
Figure 16: WT supernatant after five days of growth has significantly elevated protease activity. Protease activity was assessed through a casein fluorescein assay. Fluorescence was measured at 519 nm. Mean values from 3 repeats were plotted with error bars depicting S.E.M.

M9 media control; the sole exception being the WT supernatant from 5 days of growth. Data were analysed with a one-way ANOVA ($F_{(11, 23)}$ = [3.271], P = 0.0080) and Tukey's post hoc test.

3.6 Competition Assays

Mixed culture experiments were run for similar times as the log phase assays and long-term growth assays performed previously. All mixed cultures were set up in minimal media with the ratio of 97:3 WT to either *Δlon* or *ΔlonR.* All had the same total number of bacteria to begin. After 4 hrs (250 mins) and 20 hrs (1200 mins),

there was no difference in the population structure regardless of if the WT bacteria were competed against the *Δlon* or *Δlon*R strains (figure 17).
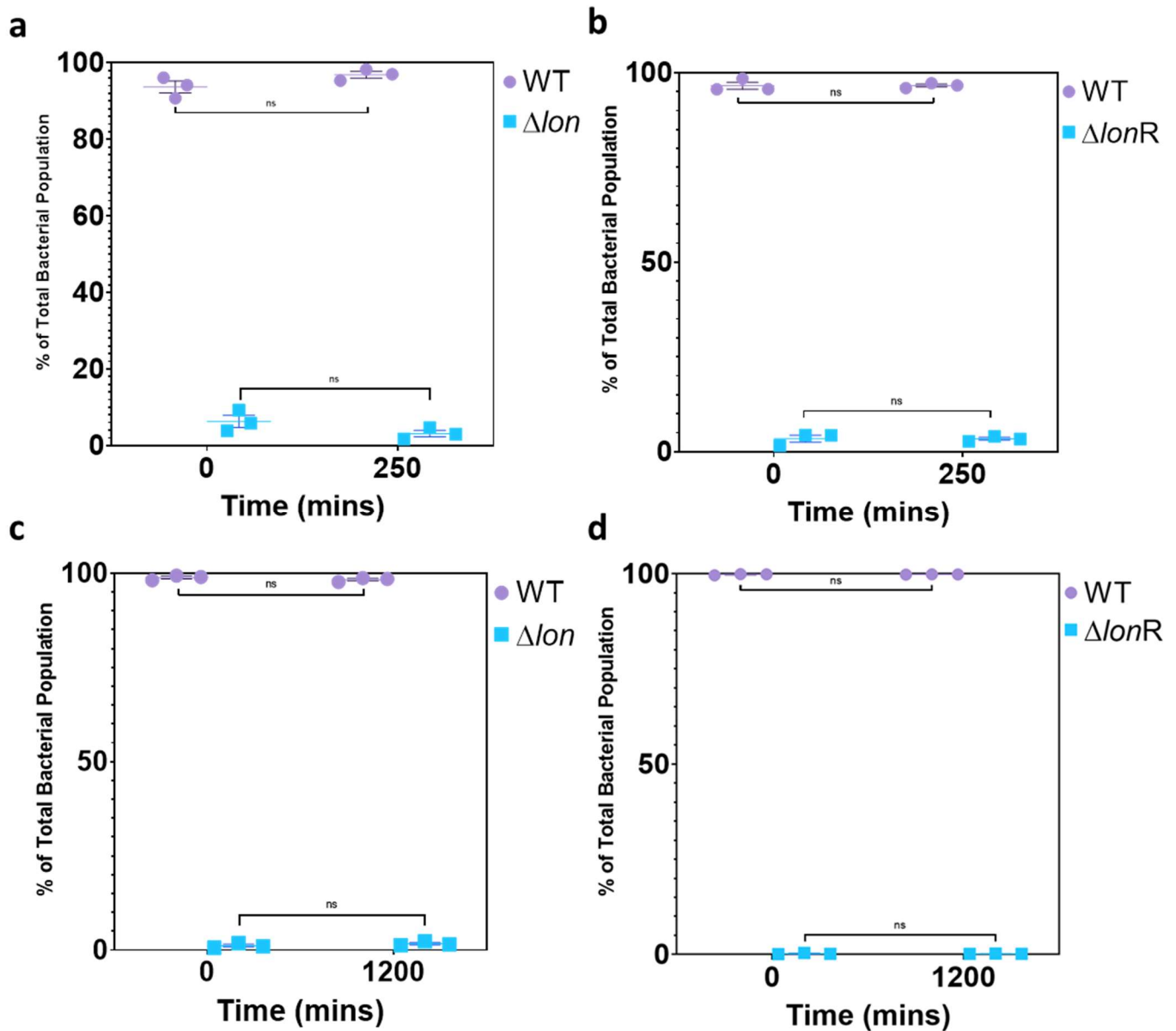
Figure 17: WT cultures grown with either *Δlon* (a, c) or *Δlon*R (b, d) in M9 media. The initial ratio was kept constant at 97:3. Viable counts with antibiotic separation were used to determine actual start and end percentages of each culture. After 250 mins (**a**, **b**) and 1200 mins (**c, d**) of growth, there was no change in the proportions of the culture. 3 independent repeats are shown. Error bars represent S.E.M.

After five days, however, as the bacteria start to suffer the effects of nutrient stress, the *Δlon* strain (figure 18a) does begin to 'cheat', increasing their frequency in the population to approximately 10%. The data were analysed using a two-way ANOVA

followed by Šidák's multiple comparisons test. There was no similar effect when observing the *Δlon*R strains (figure 18b). 'Cheating' was still occurring at 11 days, and the percentage of the population comprised of *Δlon* (figure 18c) had increased further. Once again, no effect was seen in the WT/ *Δlon*R mixed culture (figure 18d).

The WT/*Δlon* mixed cultures that grew for 21 days (figure 18e) have an extensive range in the number of viable colonies of each strain, with one replicate having 0 colonies of either strain at a x10^{-1} dilution. The repeats which did still contain viable colonies both showed a cheating effect. All replicates of the WT/*Δlon*R mixed culture (figure 18f) were also not viable by the end of the 21 days of growth. This lack of growth is despite each culture remaining alive when the strains were grown individually.

3.7 Δlon had a fitness advantage over WT when its population was small

Following on from the above experiments, the five-day time point was selected to move forward with to investigate whether the advantage, seen in the static ratio competition assays, held when the initial percentage of *Δlon* was varied. This time point was chosen as the change in fitness was best demonstrated, and the variation between the *Δlon* replicates was minimal.

WT and *Δlon* strains were grown together for five days in M9 media. The starting and final ratios were determined by serially plating the cultures onto either plain LB agar or LB agar containing kanamycin, to which *Δlon* is resistant. The starting ratios were varied between 99.9:0.1 to 70:30 WT:*Δlon*. The total bacteria in the culture were kept constant at an OD of 0.05. Antibiotics could not consistently separate ratios above

70:30 and so these were not used. This data was used to determine the relative fitness of the *Δlon* strain compared to the WT strain by comparing the initial proportions of Δ*lon* to the final proportion. A relative fitness value of 0 indicates that neither strain was selected over the five days, whereas a value of 2 shows that the mutant grows at twice the rate of the WT strain.

The calculated relative fitness values for the *Δlon* strain were plotted with respect to the $\log_{10}$ of the initial percentage of *Δlon.* The results of this analysis can be seen in figure 19. There is a negative relationship between the two variables, with the relative fitness of *Δlon* decreasing from 2.16 to –0.08 as its initial percentage increases from 0.23% to 35%. A simple linear regression was fitted through the data with an R-value of 0.8095, indicating a strong correlation. Unsurprisingly, the same pattern is observed if the WT strain's relative fitness and initial percentages are plotted (supplementary figure 5). Correlation analysis using a two-tailed parametric Spearman's correlation test revealed that this relationship was significant ($r(3)$ = -0.9608, p = 0.0093).

This data suggests that at small initial percentages of *Δlon, Δlon* does have a growth advantage over the WT bacteria. However, as the start percentage of *Δlon* increases above 15%*, Δlon* has no advantage and is actively selected against.

3.8 Expression and Purification of TonLon

The expression and purification of *Ton*Lon was performed for usage in future experiments, not within this project's scope. The total protein yield was 30 mg/mL.
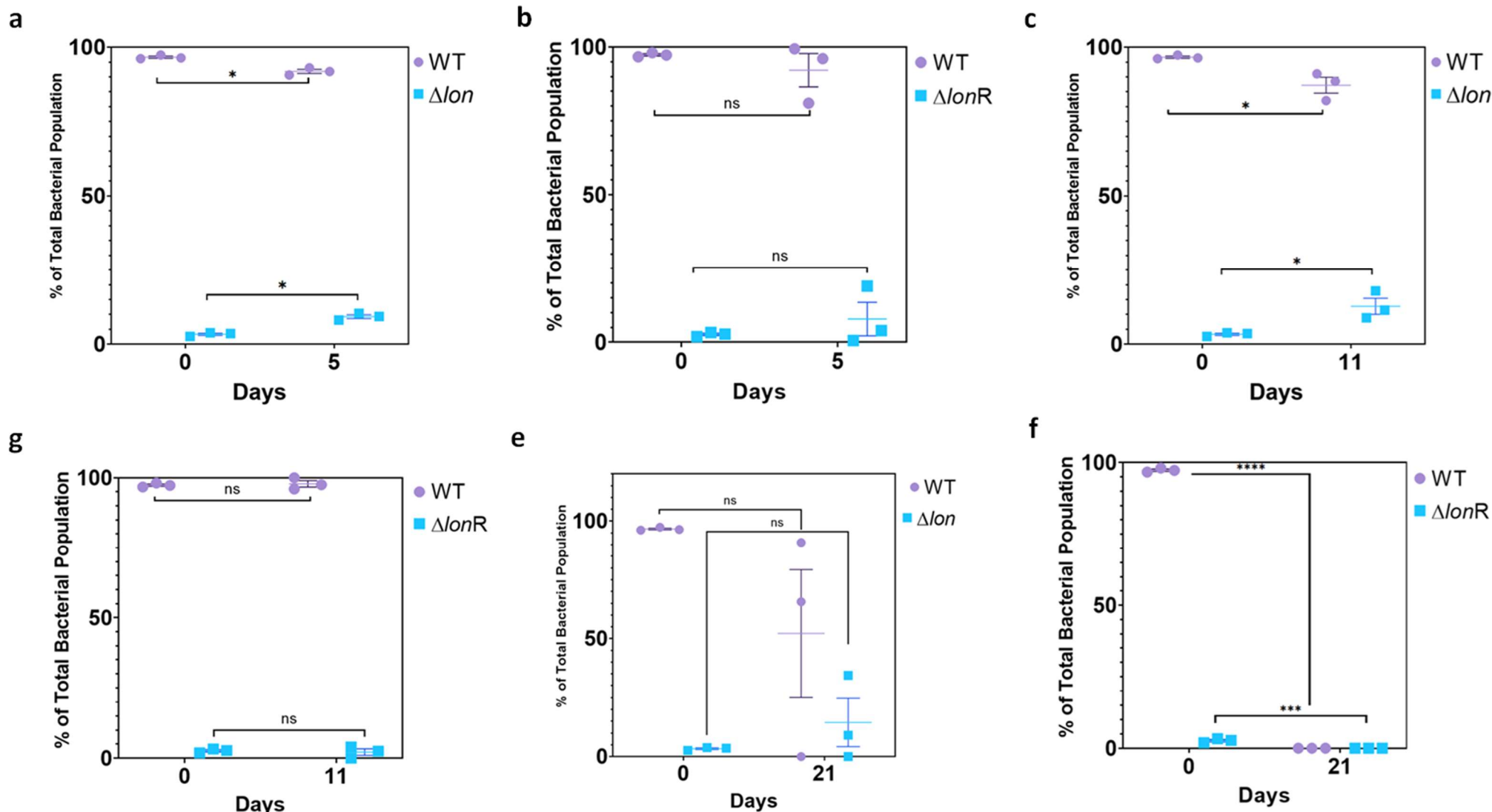
Figure 18: WT cultures grown with either Δ*lon* (a, c, e) or Δ*lon*R (b, d, f) in M9 media for 5 (a, b), 11 (c, d) or 21 (e, f) days. The initial ratios were kept constant at 97:3. Viable counts with antibiotic separation were used to determine actual start and end percentages of each culture. After 5 (**a**) and 11 (**c**) of growth, there was a significant increase in the percentage of the population comprised of Δ*lon*. The percentage increase got larger with time. There was no change to the culture's population make-up when the WT and Δ*lon*R strains were mixed apart from **f** where there were no viable colonies. 3 independent repeats are shown. Error bars represent S.E.M.
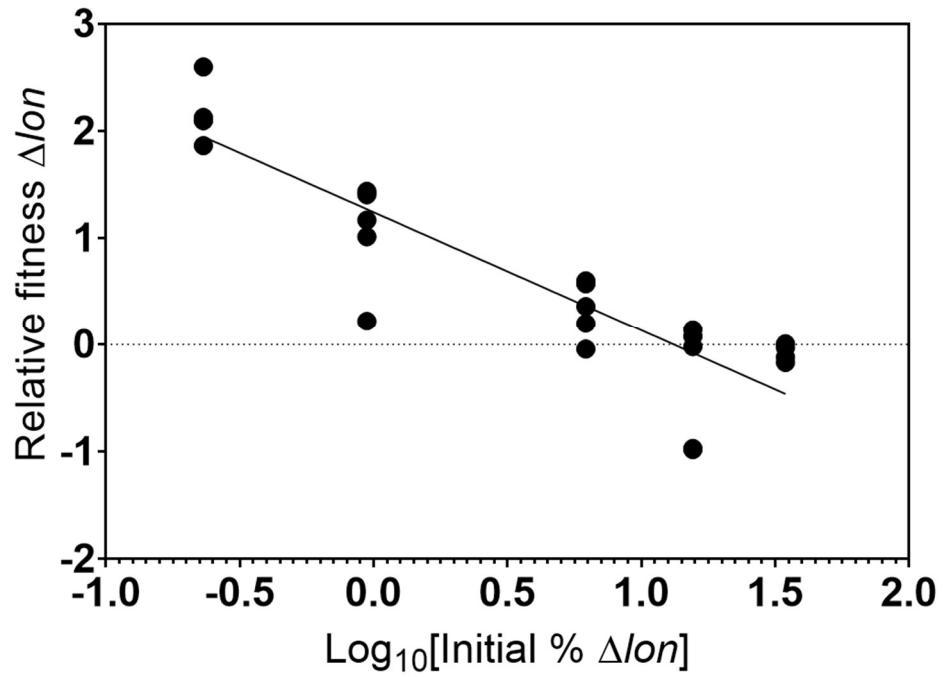
Figure 19: Relative fitness of the Δ*lon* mutant is dependent on the initial proportion of the mutant in the population. Relative fitness is calculated through comparing the growth rates of the Δ*lon* relative to that of the WT. Data (n = 5 except for 30% initial percentage where n = 4) is fitted with a simple linear regression.

4. Discussion

4.1 The In vitro long-term growth model supports a modified post-death metabolism hypothesis

Previous research by the Cann group showed that the presence of Lon in an extracellular environment led to the growing strain gaining a growth benefit. The working hypothesis is that the Lon protease, produced in *E. coli* as part of its stress response is released into the extracellular environment when the cell dies but retains some of its activity. It breaks down the proteins released from the dead cells which can be taken up by the remaining living bacteria and utilized as nutrients to alleviate stress (Payne and Gilvarg, 1968). The first aim of this project was to create an *in vitro* model of post-death metabolism. This model could then be used to test predictions made by the working hypothesis.

4.1.1 The WT strain is capable of creating and using peptides, unlike the Δlon.

BCA assays measure the total protein content of a sample, including any peptide chains above a tripeptide in size (Wiechelman, Braun and Fitzpatrick, 1988). The Pierce assay is a modified BCA assay designed to detect the peptide concentration of a sample (Thermo Fisher Scientific, no date). Details of this assay are unknown including the size of peptides which can be detected. It can be inferred, however, that the lower size limit for detection (a tripeptide) remains the same.

When analysed with the Pierce assay, there was an obvious pattern across the WT supernatants. The peptide concentration significantly decreased from its peak at days 10 and 15 to the final point at day 25 whilst the peptide concentration in the *Δlon* extracellular environments was consistent. A BCA assay was then performed to

ensure that this discrepancy was not due to differences in total protein released by cell lysis. However, the WT and Δ*lon* extracellular environments were found to have similar total protein concentrations at all time points.

The BCA assay did show that the protein concentrations in the WT environment peaked after five days of growth and then decreased from the peak to the 25-day time point. After 25 days of growth, there is a large difference between the mean protein concentrations in the WT and Δ*lon* environments – although this was not statistically significant. Since, the variability in how the cultures behaved increased the longer they were grown for, as they became more genetically distinct, increasing the number of replicates may reduce any noise and allow a clearer pattern to emerge. Additionally, since the BCA assay measures total protein concentration, any changes to the concentration of small peptides alone may be masked by the large number of proteins present.

For the post-death metabolism hypothesis to be correct, the Lon protease must break down the protein contents of the dead cells into short-chain peptides which can be taken up by the living cells. The given size limit in the literature for a peptide to be imported into *E. coli* is 650 Da, approximately equivalent to a five (or six if very small) amino acid long chain, although this depends on which amino acids are imported (Payne and Gilvarg, 1968). The above results are consistent with this prediction. Both strains release a comparable amount of protein into their extracellular environments at every time point. Only in the WT, however, are the proteins digested by Lon into short enough peptides to taken up by the living cells. This explains the decrease in peptide concentration as time proceeds. No such change is seen in the peptide

concentration of the Δ*lon* extracellular environments as the lack of Lon means no nutrient import can occur.

4.1.2 Lon protease activity in the extracellular environment is variable.

The use of FITC-Casein to measure the difference in proteolytic activity between the WT and Δ*lon* extracellular environments was mainly unsuccessful. As was expected, no difference was observed before or after day five. This result immediately suggests that Lon is only active in the extracellular environment for a short time. However, this is inconsistent with this project's previous foundation work, which showed Lon's growth benefit on growing strains within 20 hours.

The FITC-Casein assay was developed to measure the proteolytic activity of potent proteases such as trypsin (Twining, 1984). It was not designed to be used to analyse supernatants for protease activity, and there is no record in the literature of it being used for this purpose previously. With optimisation, this method could probably be validated for this purpose and used to yield improved results. However, due to time constraints, such optimisation was not achievable. Time constraints also meant that only half of the time points could be investigated, and these had only three biological replicates. Ideally, this experiment would be repeated with at least six replicates as, especially at the later time points, the cultures have become highly distinct and behave very differently. A minimum of six replicates would help reduce the variability from the biological replicates and the assay noise.

An alternative explanation for the peak in activity at day five may involve the various mechanisms through which Lon can act. The long-term growth experiments show that after five days of growth, the WT is nearing the end of the death phase. The

maximum number of cells have died and been lysed, releasing the contents into the extracellular environment – including both Lon and ATP. Although ATP will only transiently be at a high enough concentration, Lon may be able to use its ATP-dependant proteolytic mechanism which is far stronger and more effective (Gottesman, 1996). This is in contrast to the majority of post-death metabolism which has to rely on the far weaker ATP-independent mechanism, and so, may explain the spike in activity.

4.1.3 The Δlon strain has an extended lag phase compared to the WT.

It is difficult to explain why the *Δlon* strain had a slower growth rate in the lag and early log phases. Some literature has suggested that *lon* may be upregulated during the lag phase to help remove and replace damaged proteins (Rolfe *et al.*, 2012). This has not been fully substantiated but may partially explain the growth delay. The *Δlon* cells would have to contend with the adverse effects of misfolded and damaged proteins. This would slow down their protein production rate as chaperone proteins cannot pass misfolded proteins on, preventing the chaperones from folding nascent proteins. *E. coli* do have other proteases, such as ClpXP which can perform similar roles to Lon, which is likely why after a short delay the *Δlon* strain does enter exponential growth (Gottesman, 1996; Schlieker, Bukau and Mogk, 2002; Baker and Sauer, 2012).

4.1.4 The Δlon strain does not appear to enter a stationary phase.

The original post-death metabolism hypothesis also does not explain why *Δlon* does not appear to enter a stationary phase. Whilst the stationary phase may just be delayed, it may also be that the presence of extracellular Lon - or more likely the small

amino acid chains it produces - acts as a signal to the cell that nutrients are scarce. Nutrient scarcity is one of the major signals for a bacterial population to move from the log phase to the stationary phase (Notley and Ferenci, 1996). Thus, without this signal to the population, *Δlon* cells either delay making or never transition out of the log phase. This results in the apparent growth advantage of the *Δlon* strain over the WT, seen in both the OD curves and the one-day long-term growth assays.

4.1.5 The Δlon bacteria do not enter the long-term stationary phase.

The long-term growth assays were designed to investigate whether the absence of the Lon protease eliminated cryptic growth and prevented entry into the long-term stationary phase. The post-death phase is characterised by the bacterial population remaining at a low but consistent level (Zambrano *et al.*, 1993; Finkel and Kolter, 1999; Finkel, 2006).

The long-term growth assays confirmed what had been observed in the short-term growth assays above; namely that the *Δlon* strain has a significant growth advantage after one-day of growth, presumably due to its delayed entry into the stationary phase. Additionally, however, there appears to be a difference in how and when the *Δlon* strain enters the death phase. Whilst *Δlon* does die off between one and 10 days, the amount of death is not as severe as that of the WT strain in the same time period and there remains a significant difference between the two populations. This is likely due to the delayed stationary phase entry. The lack of signal to the cells to slow down their growth could mean that the cells keep trying to replicate, despite the lack of nutrients, leading to a reduction in the percentage of the population which died off.

After 10 days of growth, the WT bacteria have entered the fifth growth phase – the long-term stationary phase. It remains in this phase until at least day 25 and presumably beyond had the experiment not been concluded. The *Δlon* strain does not show any signs of entering this phase. Indeed, some individual replicates showed no viable colonies after 25 days of growth, indicating that they could not enter the long-term stationary stage and, instead, the whole population died off.

Overall, this result lines up well with the predictions of the post-death metabolism hypothesis. Namely, that the WT strain appears able to survive the severe nutrient stress it is under by recycling the dead bacteria through the action of the Lon protease. The *Δlon* cannot, leading to the population crash seen by day 25, which presumably could not be recovered without the addition of nutrients into the environment.

4.1.6 An updated model of Post-Death Metabolism.

Overall, the findings provide support for post-death metabolism. However, taken together, they reveal the possibility that Lon is not just the protease involved in protein recycling but may also play a greater role in regulating the phases of bacterial growth.

An updated model of post-death metabolism is, therefore, proposed. When cells grow in the log phase, nutrients and resources are freely available. However, as they run out, some cells in the population start to die, releasing Lon into the extracellular environment. Lon digests the dead cells' protein matter, producing small peptides that can be taken up by the living cells to supplement the nutrients. This import of the small peptides is theorised to signal to the live cells to move into the stationary

phase, where the growth is slowed. However, without this signal, entry to the stationary phase would be severely delayed. The *Δlon* population, therefore, continues to grow - recognising neither the nutrient stress it is under or that growth needs to be slowed to conserve nutrients for the good of the population's survival. What appears to be a growth advantage is a disadvantage to the population's overall survival, and soon, the population numbers decrease.

Despite the continued nutrient recycling in the stationary population, eventually, all nutrients run out. The WT population enters the death phase and, subsequently, the long-term stationary phase. In this phase, nutrient recycling is essential as there is no other source of nutrients and amino acids, so the recycled building blocks are all the few remaining living cells have to live on. It is an effective system, however, as cultures have been known to exist in this phase for years until the famine is over. The *Δlon* strain, which cannot perform this recycling of amino acids, continues to crash irrecoverably.

4.1.7 A potential signalling pathway.

One of the best studied factors which regulate the entry to stationary phase is the sigma factor σ^S and the gene that encodes it (*rpoS,* sometimes called *katF*). σ^S is known to control the general stress response in *E. coli* (Lange and Hengge-Aronis, 1991b; Loewen and Hengge-Aronis, 1994; Hengge-Aronis, 1999). It is produced in response to nutrient stress amongst other forms and is an essential factor in inducing the morphological and metabolic changes that occur during the entry to stationary phase (Hengge-Aronis, 1993; Jishage *et al.*, 1996). These changes include the *bolA*

gene becoming active, causing the cells to become more spherical, amongst many others (Lange and Hengge-Aronis, 1991a).

The revelation that the presence of the Lon protease may also regulate entry into the stationary phase means a connection between it and σ^S should be considered. The possibility of a link between the Lon protease and σ^S has been partially evaluated before. The literature is not conclusive about whether such a relationship exists, with reports that Lon has no effect and that Lon has a negative impact on the activity of σ^S (Ranquet and Gottesman, 2007; Van Melderen and Aertsen, 2009).

However, these studies only looked at the effect of the Lon protease on the activity of σ^S and do not consider the possibility that any link may work the other way around. Whilst Lon can be produced independently of *rpoS* activity, it may be that the presence of σ^S in the RNA polymerase is one of several signals that can promote the transcription of the *lon* gene. This could create a feedback loop where induction of *rpos* leads to the activation of *lon*. Lon then creates the small chain peptides which act as a signifier of the level of nutrients in the extracellular environment which informs the cell as to whether the σ^S controlled general stress response should continue.

4.2 ΔlonR is not a constitutive rescue

*Δlon*R was designed as a constitutive rescue mimicking the behaviour of the WT. However, it consistently fails to behave as a rescued phenotype despite western blot analysis confirming it produces a comparable amount of Lon to WT (see figure 8). The difference in behaviour between the two strains is particularly apparent in the long-term growth assays. The *Δlon*R strain was created by transforming *Δlon* with a

pBAD33 plasmid containing the *E. coli lon* gene. The promotor used in this plasmid was designed to be activated with L-Arabinose, of which a small amount is naturally produced by the cell. This leads to a low level of Lon being constantly transcribed without the need for any induction. This stress-independent production is very different from the stress-induced production of Lon in the WT strain. The presence of Lon under non-stressful conditions is likely responsible for the differences in behaviour between the WT and *Δlon*R strains. The *Δlon*R strain does not have to wait until the population is sufficiently stressed to start Lon production, and so start nutrient recycling. It could be argued, therefore, that the results of the *Δlon*R growth experiments, with its higher background levels of Lon as production has started earlier, may predict how the WT would behave if grown for a more extended time.

4.3 Δlon can 'cheat' and exploit the pooled nutrient resources

4.3.1 The Δlon strain has a fitness advantage over WT only when at a low population.

Whilst the *Δlon* strain is not adapted for improved nutrient scavenging, it did have a growth advantage over WT as it enters the stationary phase (after approximately after five days in M9 media). This growth advantage was short-lived, however, as it had disappeared by 25 days of growth but still could indicate that *Δlon* confers a GASP phenotype onto the cells.

One of the hallmarks of GASP phenotypes is that they can outcompete WT bacteria during the stationary phase. When looking at mixed WT/*Δlon* culture grown for various time points up to 21 days, the *Δlon* was seen to be able to outcompete the WT after five days. However, by 21 days, the advantage had disappeared. The variance in the day-21 result is far greater than at any proceeding time point and is

especially exacerbated by the replicate which had no viable colonies of either strain. However, as the Δ*lon* could not sustain its ability to outcompete the WT, it is unlikely that it constitutes a GASP phenotype.

Two other hypotheses could conceivably explain this observation. One, that the initial growth advantage and belated death phase meant that the Δlon cells simply remained in the population for longer. The other plausible explanation of the findings was that the Δlon population could take advantage of the environment and 'cheat' the WT bacteria present of nutrients and resources. The two hypotheses were tested by varying the initial start percentage of Δ*lon.* If the first hypothesis proved correct, the relative fitness of the Δ*lon* should have remained constant as it should not be a factor of the percentage in the population. However, a negative correlation was discovered between the initial percentage of Δ*lon* and its relative fitness compared to the WT.

This finding was not unexpected. Population-wide systems such as quorum sensing have long been known to be vulnerable to cheaters. It is advantageous for the cheater not to incur the energy cost of producing the common signal but still gain from the benefits. However, if the population becomes too full of cheaters, the whole population is negatively affected as there aren't enough common goods/signal for every cell (Axelrod and Hamilton, 1981; Wilson, Pollock and Dugatkin, 1992; West, Griffin and Gardner, 2007). In this instance, the WT cells after they have died have released any Lon present in them into the extracellular environment to break down the protein content of the dead cells into peptides which are then a common nutrient resource which all cells can use. The Δ*lon* population would be able to 'cheat' this

system and use the nutrients (the peptides) created by the WT Lon whilst not having to incur the energy cost or deal with the potentially harmful side effects of producing the Lon protease itself.

This interpretation explains the inverse relationship between the initial percentage of *Δlon* and its relative fitness because, as there is more *Δlon* in the population initially, fewer cells produce Lon to be released into the environment when they die. This means fewer short-chain peptides are created, and so the nutrient stress is exacerbated. This is likely why one of the replicates was wholly dead by day 21 when the WT cultures could survive for 25 days plus when grown alone - the pressure of the competition and the stress caused by the 'cheating' *Δlon* proved too much for the culture to handle.

4.3.2 How did the post-death activity of Lon evolve?

At its most simple, the process of evolution occurs over three steps: there is natural genetic diversity in a population; selective pressure is applied, and the genetic diversity means some of the population are better suited to survival under these conditions; the organisms which survive, pass their genes down to their offspring becoming more common in the population as the trait is selected for. Altruistic behaviour, such as post-death metabolism, is favoured when directed at highly related nearby individuals (kin) as it increases the likelihood of those shared genes being passed on (Hamilton, 1964; Diggle *et al.*, 2007; West, Griffin and Gardner, 2007).

However, the activity of Lon in post-death metabolism presents a conundrum. How can this activity be selected for when the advantage it confers happens after death

and, therefore, after reproduction? Furthermore, how is the altruistic post-death metabolism still favoured when no known mechanism directs the benefits towards close kin?

A solution to this is that Lon, and any other machinery involved in this system, must have first evolved an important function in life causing it to be selected for. Lon was first discovered as a heat-shock protease and is known to have an essential function in *E. coli,* especially when the cells are under stress (Phillips, VanBogelen and Neidhardt, 1984). The role in post-death metabolism then came later. Once the post-death activity was established, communities which had post-death Lon activity would be better adapted to surviving stress, thus, outcompeting those which did not. Additionally, the social benefit of post-death metabolism is so important to a stressed population, and the additional cost to the individual so low, that it far outweighs the relatedness factor causing the behaviour to be selected.

4.3.3 How are 'cheaters' prevented in the population?

The essential role Lon plays during life is also potentially how cheaters are prevented in the population. Lon is important for individual and group survival during stress. Hence, the benefit of not producing Lon, and relying on neighbouring cells for nutrient recycling, does not outweigh the disadvantage the cheater would face when trying to grow under the same stress.

Furthermore, since Lon is already being produced in the cell due to its role in the response to stress, its post-death role is not a detriment to the cell. There is no additional cost to the cell, especially since it is already dead when this activity occurs.

4.4 Future Work

Many of the experiments discussed above were in some way limited in their scope by the time constraints on this project. For example, a long-term growth assay where *Δlon* would be grown for 50 days would show conclusively whether this strain can enter the long-term stationary phase. Further development of the FITC-Casein assay into a reliable method for use with supernatants and low-activity proteases would also validate the activity of Lon in an environment lacking in ATP. This method could also then be used to ascertain whether there is a limit to how long the Lon can remain active for in this unconventional environment. In addition to continuing the experiments already discussed, there are an abundance of unanswered questions raised by this study and numerous directions in which research could progress from here.

4.4.1 Does Lon have a greater role to play in regulating bacterial growth phases?

This study provided evidence that Lon (or its products) appears to play a role in the regulation of bacterial growth phases, specifically regulating the entry to the stationary phase and potentially the long-term stationary phase. A potential link with the sigma factor encoded by *rpos* has been discussed above, which may be worth exploring further. However, the data is far from conclusive and any regulatory role would have to be further investigated, not least as it is yet unknown whether a lack of Lon delays the stationary phase or if its presence is key for entry to the phase altogether.

4.4.2 Do other enzymes have complementary roles in post-death metabolism?

It is accepted that bacteria in the stationary phase recycle degraded proteins to gain amino acids, although the mechanism was not understood until now. However, a dead and lysed cell would yield more usable biomolecules than proteins alone, such as genetic material, lipids, and peptidoglycans, all of which could be digested for their monomers. Whilst this project only focused on the breakdown of the protein content, it is likely that a range of nucleases, lipases and other catabolic enzymes would also function as part of the post-death metabolic pathway. A genetic screen may reveal several candidates for these roles. Any such screen should focus on candidates that fulfil the following criteria: perform an essential function in life, have a wide range of targets, and have an ATP-independent mode of action.

4.4.3 Is this post-death metabolic pathway evolutionarily conserved across species?

In nature, a bacterial species rarely exists in isolation. A lack of available nutrients in an environment will affect all species present, although each species may have different mechanisms to help survive the famine. Future work is required to understand whether species other than *E. coli* undergo post-death metabolism. This would be especially interesting in species such as *Bacillus subtilis,* which usually adopts an endospore form during nutrient stress and the stationary phase (Chubukov and Sauer, 2014). If post-death metabolism is present, extracellular Lon/short-chain peptides may be one of many signals which promotes sporulation, or it may be an alternate stress response entirely. As Lon is so ubiquitous across life, this exploratory research could be extended to unicellular eukaryotic organisms.

4.4.4 Could this be an example of cross-species altruism?

Should post-death metabolism be a typical adaptation, the question of whether the peptides produced can be shared between different species then needs to be addressed. It is clearly beneficial for a species to keep all recycled nutrients for its own use and outcompete a more cooperatively minded species. That being said, it is difficult to imagine how a promiscuous protease like Lon, which does not cleave polypeptides into specific peptides, could create peptides which could only be imported by the same species which produced the Lon. Regardless, research into whether the benefits of post-death metabolism can be observed between species would improve understanding of how bacterial competition works in shared niches.

4.5 Future Applications

4.5.1 A novel approach to treating biofilms.

Biofilms are an increasing problem in modern medicine. They show up on medical equipment such as implants and catheters and are responsible for many of the secondary nosocomial infections, affecting up to 20% of NHS patients (Ledwoch *et al.*, 2018). They are also often the cause of chronic infections. For example, *Pseudomonas aeruginosa* causes persistent lung infections in cystic fibrosis patients, as they are highly resistant to antibiotics and other antimicrobial treatments (Donlan and Costerton, 2002; Høiby, Ciofu and Bjarnsholt, 2010).

Biofilms are a survival mechanism for many bacterial species, with up to 80% of all known species being able to form these structures, including *E. coli* (Flemming and Wuertz, 2019). Biofilms form through steps regulated by quorum sensing (Davies *et al.*, 1998). Simply, cells from either one or many species, adhere to a surface and

create an extracellular matrix surrounding them (Muhammad *et al.*, 2020; Uruén *et al.*, 2020). This existence leads to a complex and highly stratified community, where the cells on the outside have far greater access to oxygen and nutrients, allowing them to proliferate, whilst the deeper cells grow at a sluggish pace (Stewart and Franklin, 2008; Ciofu *et al.*, 2022)). Nutrients, especially amino acids, are still supplied to the inner cells by nutrient recycling due to post-death metabolism (Blenkinsopp, 1991; Mashburn *et al.*, 2005; Thi, Wibowo and Rehm, 2020).   These inner cells are often the most resistant to the action of antimicrobials (Blenkinsopp, 1991; Werner *et al.*, 2004; Stewart and Franklin, 2008). Lon is also known to be essential for several species to form a biofilm successfully, induce the genetic changes needed to form persister cells, and achieve full virulence (Breidenstein *et al.*, 2012; Rogers *et al.*, 2016; Patel, Buchad and Gajjar, 2022).

There is potential, therefore, for drugs which target the activity of Lon to be successful at helping treat biofilms. Such drugs could be used in conjunction with existing or novel antimicrobials in order to effectively target both strata of the biofilm at once.

4.5.2 Potential for improving waste treatment processes.

Biofilms are also regularly found in waste treatment centres, although in a positive context. These centres produce large amounts of sludge, which is starting to threaten human health if not properly treated. This sludge is also incredibly expensive to treat and make non-hazardous (Zhang *et al.*, 2009). Current methods include heating the sludge with microwaves, ultra-sonication, and pre-treatment of the sludge to enhance degradation using cell-lysis technologies (Zhang *et al.*, 2009; Tyagi and Lo, 2011; Romero *et al.*, 2013; Wang *et al.*, 2015). During this pre-treatment, microbes

are added to the waste product, which lyse any cells present. These microbes then metabolise the waste, organic matter, and cell lysis products to reuse the nutrients for their growth (Tyagi and Lo, 2011). Despite being widely used, the main problem with pre-treatments is its slow hydrolysis reaction, specifically the rate-limiting step (Jang and Ahn, 2013; Wang *et al.*, 2015). Therefore, increasing the expression of Lon in these microbes or selecting strains more efficient at post-death metabolism could have a positive impact this degradation. The higher concentration of Lon would mean that for the same volume of microbes added, more sludge can be digested, and the efficiency of this process improved.

4.5.3 Additional targets for improved biofilm formation

Waste processing is not the only positive context in which biotechnology uses biofilms. Biofilms have been found to protect against corrosion and rust formation (Zuo, 2007). Biofilms may also be helpful in bioremediation – where toxic compounds are broken down into harmless ones either *in situ* or *ex situ* – and benefit plant growth (Upadhyayula and Gadhamshetty, 2010). Biofilms from various rhizobacteria already colonise many plant roots, protecting the plant from infection whilst providing much needed nitrates in return for sugars. There is, therefore, interest in using these beneficial strains and incorporating them into fertilisers (Muhammad *et al.*, 2020). Improving the biofilm's ability to use its available nutrients may also improve its ability to perform the desired outcomes.

5. Conclusion

In conclusion, the Lon protease in *E.coli* plays an active and essential role in post-death metabolism. This thesis has proposed an updated model of post-death

metabolism. Lon is responsible for both degrading protein content, when released from a dead cell into the extracellular environment, thus creating peptide chains which can be imported into living cells to alleviate nutrient stress. It also potentially acts as part of a regulatory pathway controlling the entry of a living cell into the stationary phase, although any mechanism is speculative. Additionally, this project demonstrated that *Δlon* cells have a fitness advantage over the wild-type and will exploit their cryptic growth when possible.

The identification of the Lon protease as a key player in cryptic growth leads to the possibility of other proteins with complementary roles. Hopefully, this will lead to the mechanisms underpinning cryptic growth to be finally unravelled. Finally, discussions around how such a mechanism could possible evolve may help further inform the conversation around altruistic bacteria and the various selective pressures they face.

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