High level indole signalling in *Escherichia coli*

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Title

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Abstract

Indole is a small signalling molecule, produced by many species of bacteria, including *Escherichia coli.* It is made by the enzyme tryptophanase, which converts tryptophan into indole, pyruvate and ammonia. Indole has diverse roles in *E. coli,* including regulation of biofilm formation, acid resistance and pathogenicity. In these cases, *E. coli* responds to a low, persistent level of indole (0.5-1 mM), similar to the concentration found in an *E. coli* culture supernatant in stationary phase (typically 0.3-0.8 mM). Recently, it has been shown that much higher concentrations of indole (3-5 mM) inhibit cell division by acting as an ionophore to dissipate the membrane potential. However the biological relevance of such high concentrations, and therefore these aspects of indole signalling, has been questioned. This work has investigated the role of indole signalling during entry into stationary phase, when indole production is quickly upregulated. The viability of non indole producing mutants was compared to wild-type indole producing cells. In the short term indole producers suffered a growth disadvantage, but in the long term they were significantly more viable than their indole non-producing counterparts. The addition of 1 mM indole to the indole non-producing culture failed to complement the phenotype. A hypothesis was developed that a high rate of indole production during stationary phase entry leads to a transient, high concentration of indole inside the cell. This regulates cell growth and division *via* the ionophore mechanism. The validity of this indole pulse signalling hypothesis was tested by measuring cell-associated indole. For a brief time during stationary phase entry cell-associated concentrations reached 60 mM. Cell-associated indole represents an average of indole in the cytoplasm and the cell membrane. It was shown that indole has an approximately 100-fold greater affinity for the cell membrane. 60 mM cell associated indole is equivalent to approximately 4 mM in the culture supernatant, suggesting that the indole ‘pulse’ is sufficient to inhibit growth and cell division on entry into stationary phase. The indole pulse was dependent on the stationary phase sigma factor, SigmaS, which increases tryptophanase expression on entry into stationary phase. This increased tryptophanase expression occurs immediately prior to increased indole production. The end of the pulse seems to correlate with the exhaustion of tryptophan in the growth medium.

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. The length of this dissertation is no more than 60,000 words.

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Abbreviations

AHL acetylated homoserine lactone

AI-2 auto-inducer 2

AIP auto-inducing peptide

ATP Adenosine triphosphate

A/E attaching and effacing

cAMP cyclic adenosine monophosphate

CRP cAMP receptor protein

CCSM cell to cell signalling molecule

c-di-GMP cyclic dimeric guanosine 3’-5’-monophosphate

cGMP cyclic guanosine 3’-5’-monophosphate

CSP competence-stimulating peptide

DFS cis-11-methyl-2-dodeconenoic acid

DNA Deoxyribonucleic acid

EDTA Ethylenediaminetetraacetic acid

GF germ free

GFP Green fluorescent protein

H-NS histone-like nucleoid structuring

IAA indole-3-acetic acid

ICA indole-3-carboxaldehyde

LEE locus of enterocyte effacement

mRNA messenger ribonucleic acid

NADH/NAD+ Nicotinamide adenine dinucleotide

OD_{600} Optical density measured at 600 nm

PCR Polymerase chain reaction

ppGpp guanosine 3’-5’ tetrapyrophosphate

pppGpp guanosine 3’-5’ pentapyrophosphate

RBS ribosome binding site

Rcd regulator of cell division

RNA ribonucleic acid

sRNAs Small RNAs

TAE Tris-acetate-EDTA

UTR untranslated region

Chapter 1

Introduction

1.1 Bacterial Communication and Signalling

Bacteria are able to produce and use a wide variety of chemical signals to communicate. They are able to utilise these messages to better respond to changing environments. Signals can be sent not only to other bacteria within the same population (intraspecies signalling), but also to different species of bacteria (interspecies signalling). In some cases signals can also be sent to the eukaryotic host, as an example of inter-kingdom signalling (for recent review see Bandara *et al*., 2012). The main focus of this thesis is indole signalling in *Escherichia coli,* but in order to provide context for this small molecule and its functions, other types of bacterial signalling molecules and the roles they play in bacterial physiology will first be addressed.

The notion of bacterial communication originated in 1965, following work by Tomasz on genetic competence in *Streptococcus pneumoniae* (Tomasz, 1965). It was shown that differentiation into the competent state was determined by an extracellular factor, made by the bacterium itself, which was later demonstrated to be a modified peptide (Lazazzera and Grossman, 1998, Dunny, 2007). For almost 20 years after this, it was assumed that signalling was limited to a few specific species of bacteria, but it is clear now that bacterial signalling is a widespread phenomenon.

1.1.1 Quorum sensing

Quorum sensing is a well-studied, classical example of bacterial signalling. This term was first used by Fuqua *et al.* (1994) to describe a type of cell-to-cell communication, in which the expression of target genes is modulated in a cell density dependent way. The system is triggered when a critical concentration of a diffusible signal molecules or autoinducer is achieved in the local environment (for review see Bassler and Losick, 2006).

Although the term quorum sensing was not coined until 1994, this type of behaviour had been observed in bacteria long before this. Nealson (1977) demonstrated that bioluminescence in the marine bacteria *Vibrio fischeri* and *Vibrio harveyi* occurred only when the bacteria were present at high density. It was also shown that

bioluminescence could be induced in low density cultures by the addition of cell-free culture fluid. This became an important part of the quorum sensing paradigm, that conditioned or spent medium can be added to low density cultures to mediate the effect seen in high density cultures. The quorum sensing system controlling bioluminescence in *V. fischeri* has been extensively studied since this discovery, with the signal produced being identified as acetylated homoserine lactone (AHL) (Eberhard *et al*., 1981).

1.1.1.1 Types of quorum sensing

Generally, Gram-negative bacteria use AHLs in quorum sensing, whereas Gram-positive bacteria utilise small peptides. These classes of molecules are both made within the cell, but are able to diffuse or to be actively transported out of the cells. A fundamental principle of quorum sensing is that bacteria make the chemical signal in a population density dependent manner. As the population of bacteria grows, the external concentration of the signal molecule will increase proportionally. Eventually, the signal will reach a threshold concentration, which each individual cell within the population will detect and respond to. Detection and response requires signal transduction back into the cell across the cell membrane, which is achieved by either by direct diffusion or *via* a membrane protein. This then results in a signalling cascade being triggered inside the cell, resulting in altered gene expression.

An AHL type circuit typical of those found in many Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Vibrio fischeri,* is shown in Fig. 1.1A. The circuit typically consists of a LuxI type enzyme, which synthesises the AHL molecule (Shaefer *et al*., 1996). AHL molecules consist of a homoserine lactone ring, attached to a variable length acyl chain, *via* an amide bond (Reading and Sperandio, 2006). This chemical structure means that the AHL can freely diffuse across the membrane into the surroundings (Kaplan and Greenberg, 1985). All bacteria within a population produce AHL simultaneously which results in the AHL increasing in proportion to the cell density. When a sufficiently high level is reached, the AHL is able to interact with the N-terminal domain of the LuxR type receptor (Hanzelka and Greenberg, 1995). The complex of AHL and LuxR then acts to modulate expression of target genes.

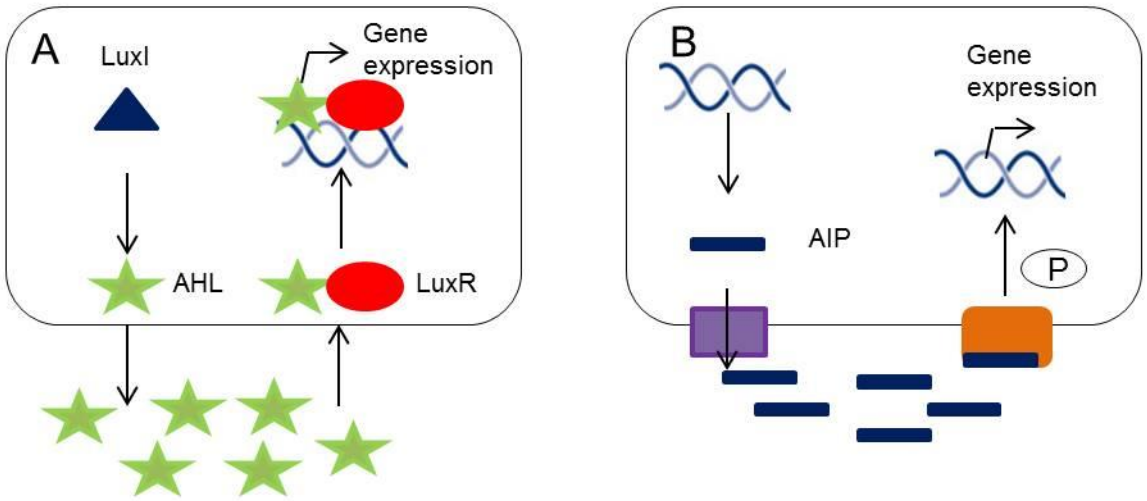


Figure 1.1 Paradigm quorum sensing circuits in Gram negative (A) and Gram positive (B) bacteria. Gram negative bacteria utilise AHL molecules made by LuxI type enzymes AHL molecules are made in a cell density dependent manner, and diffuse across the cell membrane. When a sufficient concentration is reached the AHL binds to a receptor LuxR The LuxR-AHL complex is then able to modulate expression of target genes. Gram positive bacteria use AIP molecules, which require transport out of the cell via a protein where they can bind to histidine kinases on the cell surface. This induces a phosphorylation cascade which activates transcriptional regulators allowing induction of target gene expression.

Gram-positive bacteria, such as *Staphylococcus aureus* and *Enterococcus faecalis,* typically use small, modified peptides (sometimes referred to as auto-inducing peptides, AIPs (Sifri, 2008)) in quorum sensing, also shown in Fig. 1.1B. AIPs are made as precursor peptides, typically 5-17 amino acids in length, which can undergo modification (Lazazzera and Grossman, 1998). The chemistry of the AIP molecules means that the cell membrane presents a barrier to AIP diffusion (Federle and Bassler, 2003). Therefore the AIP must first be actively excreted *via* a protein transporter (Schauder and Bassler, 2001) and then detected, not intracellularly, as AHLs are, but on the cell surface. AIPs bind to histidine kinase sensors on the cell membrane (Lyon *et al*., 2002), which induce phosphorylation of other transcriptional regulators, allowing gene expression to be modified.

In addition to these Gram-negative and positive specific systems, a system exists which has been referred to as a universal bacterial signal, called AI -2 (autoinducer 2). AI-2 is produced by more than 55 bacterial species, both Gram-negative and positive (De Keersmaecker *et al*., 2006). These include *E. coli* and *Salmonella typhimurium* (Surette and Bassler, 1998). AI-2 is responsible for inter-species signalling (Bassler *et al*., 1997). AI-2 production is dependent on the LuxS enzyme (Surrette *et al*., 1999), however the mode of action of AI-2 is species dependent

(Bandara *et al*., 2012). For example in pathogenic strains of *E. coli,* AI-2 modulates processes including expression of type III secretion systems and secretion of proteins (Sperandio *et al*., 1999). Furthermore, expression of genes involved in bacterial metabolism, DNA repair, nucleotide and protein biosynthesis, cell growth and division is also regulated by AI-2 (Sperandio *et al*., 2001).

1.1.1.2 Roles of quorum sensing systems

Quorum sensing systems have been shown to regulate a wide variety of processes and phenotypes in bacteria, particularly those regarded as 'social' behaviours. Early reports demonstrating that a wide variety of bacteria utilise quorum sensing encouraged the development of biosensors that could detect AHLs produced by bacteria (Swift *et al.,* 1993). These plasmid-based bioluminescent sensors have been used to detect quorum sensing systems in bacterial species including *Enterobacter agglomerans, Hafnia alvei, Rahnella aquatilis* and *Serratia marcescens* (Swift *et al.,* 1993).

Production of carbapenem antibiotics in *Erwinia carotovora* is modulated by the quorum sensing molecule N-(3-oxohexanoyl)-L-homoserine lactone (Bainton *et al.,* 1992). In *P. aeruginosa* production of an important virulence factor, elastase, is controlled by a LuxR type molecule (Gambello and Iglewski, 1991). It has also been shown that the expression of over 300 genes in *P. aeruginosa* are modulated by quorum sensing molecules, contributing to processes such as biofilm formation and pathogenicity (Davies *et al*., 1998). Quorum sensing has been shown to play a key role in virulence of pathogenic bacteria, including *S. aureus* and *Vibrio cholerae* (Bandara *et al*., 2012)

More recently, various other molecules have been identified which may work in a way which is analogous to the quorum sensing systems described above. These include DFS (cis-11-methyl-2-dodeconenoic acid), CSP (competence-stimulating peptide) and the small molecule which is the main focus of this work, indole (Bandara *et al.,* 2012). This is reflected in the increase in the number of publications on quorum sensing. Diggle *et al.* (2007) estimated that since the term was coined in 1994, over 1500 articles on the topic had been published. In 2014, a search for the term 'quorum sensing' on PubMed returned over 4500 articles. However, there is

significant debate in the literature whether all these recently identified molecules truly are *bona fide* quorum sensing systems. In a review by Winzer *et al* (2002*)* four key criteria are suggested which a molecule must fulfil in order to be classed as a cell to cell signalling molecule (CCSM). These are that the CCSM is made at a specific stage, accumulates externally and is recognised by a specific receptor. In addition the accumulation of the CCSM must generate a concerted response and the response by the cells should extend beyond physiological changes to remove or detoxify the molecule. The authors state that if a molecule fits these criteria, then it may also be part of a true quorum sensing system. Throughout the review, Winzer *et al* (2002) also stress the importance of the classical 'spent medium' experiments. In such experiments, mutants lacking the ability to produce the signalling molecule are expected to reproduce the wild-type phenotype when medium which had previously supported growth of the wild-type strain is added to the mutant culture. It is clear that quorum sensing is a widespread type of bacterial communication, but careful considerations should be given when assigning new molecules to this type of signalling.

1.1.2 Nucleotide Signalling

In addition to the quorum sensing molecules described previously, bacteria use a wide range of linear and cyclic nucleotides in order to gain information about the extracellular environment and to respond accordingly. Nucleotide signalling molecules (sometimes referred to as second messengers) such as cyclic guanosine 3'-5'-monophosphate (cGMP) were known to exist in bacteria as far back as 1974 (Bernlohr *et al.,* 1974), but it is only relatively recently that the large range of nucleotide signals and the roles they play have begun to be elucidated. Nucleotide signals are also often used to help bacteria integrate extracellular cues and quorum sensing molecules (for a recent review, see Kalia *et al*., 2013).

Three important nucleotide signalling molecules will be considered here: cyclic dimeric guanosine 3'-5'-monophosphate (c-di-GMP) and guanosine 3'-5' tetrapyrophosphate (ppGpp)/ guanosine 3'-5' pentapyrophosphate (pppGpp) (collectively dubbed (p)ppGpp) and cyclic adenosine monophosphate (cAMP). The discussion will focus on how and when they are produced and what their effects are.

1.1.2.1 c-di-GMP Signalling

A well-studied example of a nucleotide signal, produced by a wide range of bacteria, is c-di-GMP. The intracellular amount of c-di-GMP is controlled by diguanylate cyclases which produce c-di-GMP (Ausmees *et al*., 2001) and phosphodiesterases which hydrolyse c-di-GMP (Schmidt *et al*., 2005). Encoding two separate enzymes to produce and destroy the signal respectively is typical of a bacterial second messenger system. This allows precise control of the cellular concentration of c-di-GMP levels, because synthesis and breakdown are controlled by a range of factors (reviewed by Hengge, 2009).

c-di-GMP can mediate its effects by binding to effector proteins, transcription factors or riboswitches. An example of c-di-GMP interacting directly with a protein receptor can be found in *E. coli,* where c-di-GMP binds to the PilZ domain protein YcgR, which is important in regulating flagellum based motility (Ryjenkov *et al.,* 2006). The first report of c-di-GMP interacting with a transcription factor was by Hickman and Harwood (2008). It was demonstrated that in *P. aerunginosa*, c-di-GMP bound to FleQ, a transcriptional repressor of genes involved in exopolysaccharide biosynthesis, and that repression was relieved by c-di-GMP. Riboswitches which are modulated by the presence of c-di-GMP can be located upstream of the open reading frames for the enzymes which control c-di-GMP turnover (Sudarsan *et al.,* 2008) or, in some bacterial species, directly upstream of the genes controlled by c-di-GMP. There is also some indirect evidence that c-di-GMP interacts with the stationary phase sigma factor, RpoS, as in *E. coli* the expression of some enzymes which control c-di-GMP turnover is dependent on RpoS *(*Sommerfeldt *et al.,* 2009). The outcome of these various interactions is that c-di-GMP is important in modulating a range of bacterial behaviours from cell differentiation, conversion from a motile lifestyle into biofilms, and virulence gene expression. Indeed it has been referred to as the ‘master regulator of bacterial lifestyle’ (Kalia *et al*., 2012).

1.1.2.2 (p)ppGpp Signalling

(p)ppGpp is an important signalling molecule produced by a wide range of bacteria. It is produced in response to cellular stresses such as amino acid and carbon starvation, and for this reason has been coined an ‘alarmone’ (Kalia *et al*., 2012).

Many of the effects of (p)ppGpp are therefore observed in stationary phase. In *E. coli*, it is an important signal in mediating growth arrest during starvation, as demonstrated by comparison of wild-type and (p)ppGGpp deficient cells. Studies on (p)ppGpp deficient *E. coli* entering stationary phase have shown that the protein profiles are similar to growing wild-type cells. This suggests that, in stationary phase, *E. coli* which lack (p)ppGpp is still effectively 'programmed' for rapid growth, resulting in a reduced ability to deal with stress (Magnusson *et al.,* 2003, Magnusson *et al.,* 2005).

In *E. coli*, the intracellular concentration of (p)ppGpp is regulated by two enzymes. RelA has a single function, to synthesise (p)ppGpp (Justesen *et al*., 1986). RelA uses GTP and ATP to produce (p)ppGpp, which is then converted to ppGGpp. SpoT has a dual function, both to produce and hydrolyse ppGGpp (Xiao *et al*., 1991). The cell uses these enzymes to control precisely the cellular concentration of (p)ppGpp.

(p)ppGpp has wide and varied roles in the cell, and these are achieved by numerous mechanisms. (p)ppGpp can influence transcription in two ways, firstly by a direct interaction with RNA polymerase (RNAP). This interaction is facilitated by a protein, DksA, which binds to RNAP and enhances the effect of (p)ppGpp (Paul *et al*., 2005). (p)ppGpp can also affect transcription in an indirect manner, by interactions with Sigma factors. For example, (p)ppGpp acts to inhibit the binding of the housekeeping sigma factor (Sigma70) to RNAP, which permits other stress-related sigma factors (such as SigmaS, RpoS) to be bound to RNAP and allow direct transcription of genes relevant to starvation conditions (Jishage *et al*., 2002). In addition, (p)ppGpp also acts to protect RpoS from proteolysis by preventing it from coming into contact with the proteasome (Merrikh *et al*., 2009).

The effects of (p)ppGpp have also been shown to be concentration dependent. Traxler *et al.* (2011) showed that both the Lrp and RpoS regulons required ppGpp for their activation. However, activation of the Lrp regulon required only a low level of ppGpp, whereas the RpoS regulon was induced only when a high level of ppGpp accumulated. Balsalobre, (2011) likened this dose dependence of (p)ppGpp to a 'whispering' signal at low levels compared to a 'shouting' signal at higher concentrations.

1.1.2.3 cAMP Signalling

cAMP production in bacteria is catalysed by adenylyl cyclase which converts ATP into cAMP (Ide, 1969) and hydrolysis of cAMP is controlled by cAMP phosphodiesterases (Lehamn ,1960, Peterkofsky and Gazdar, 1971). Once again, having one enzyme for production of the signal and another enzyme for destruction allows precise control of cAMP levels.

A classic example of the role of cAMP in *E. coli* is in catabolite repression of the *lac* operon, where cAMP interacts with cAMP receptor protein (CRP), to allow the metabolism of lactose as a secondary carbon source when glucose levels are low. In the presence of low glucose concentrations, cAMP binds to CRP, and this complex activates transcription of the operon. Whereas, under high glucose concentrations, cAMP levels are low, leading to inhibition of expression of the *lac* operon (recently reviewed by Görke and Stülke, 2008).

In addition, cAMP plays important roles in a wide range of cellular processes including the formation of biofilms and pathogenicity in a wide range of bacteria, typically by affecting the expression of relevant genes. One example of this is found in *P. aeruginosa*, where the expression of a type III secretion system is modulated by the presence of cAMP (Diaz *et al*., 2011).

1.2 Stress responses in bacteria

Following the previous discussion, it is clear that bacteria use various molecules and systems to help integrate information about the extracellular environment. Signalling is especially important in the response to various stresses. Bacteria must both sense the stress and respond accordingly to it. Signalling molecules contribute to both aspects, both by indicating to the cells that stress is present and helping the cells react to it. Stresses experienced by bacteria can include changes in temperature, pH, and exposure to antibiotics and limited nutrient availability (for a review see Aertsen and Michiels, 2004). One approach to deal with the incoming stress is to physically move away from it, another approach is modulation of gene expression in order to increase resistance to the stress. This is typically achieved by

either Sigma factor responses or phosphorylation cascades. Many adaptive responses can be very specific to the type of stress, however bacteria also employ more global changes in phenotypes and metabolism to help deal with a wide range of stresses (Marles-Wright and Lewis, 2007).

1.2.1 Stationary phase entry as a stress response

The work described in this thesis is focused on one specific stress, nutrient limitation on entry into stationary phase. Therefore only the responses employed by cells to help cope with this particular stress will be discussed (for a review see Nystrom, 2004). When *E. coli* have access to sufficient oxygen and nutrients at 37°C, growth and division of the cells occurs rapidly, and the culture remains in a steady state known as exponential phase. When a specific resource becomes exhausted, growth and cell division slows as the cells enter a transition period between exponential phase and stationary phase. Eventually, growth and division cease completely, and the cells enter stationary phase. This state can be defined as the point where there is no further increase in the density of the culture as measured by OD_{600} values or, as suggested in a review by Navarro Llorens *et al*., (2010), by measuring the number of viable cells in the culture by plating out the culture and counting colony forming units (CFU). This allows a clearer view of the 'death phase', when the CFU values decrease significantly after approximately 3 days of culture without the addition of further nutrients (Finkel, 2006).

1.2.2 The role of RpoS in stationary phase entry

Many genes with important roles in stationary phase entry and survival have been identified (reviewed by Nystrom, 2004). The main contributor to starvation survival in most Gram-negative bacteria, including *E. coli*, is an alternative sigma factor, RpoS (Lacour and Landini, 2004, Weber *et al*., 2005). RpoS levels are subject to multiple controls at the levels of transcription and translation. Further controls which affect RpoS levels include proteolysis of RpoS (Muffler *et al.,* 1996). The outcome of this is that RpoS levels increase on entry into stationary phase or on exposure to many other stresses.

Genome wide transcription profiling of RpoS null mutants and isogenic wild-type *E. coli* cells has been performed to identify which genes are under the control of RpoS

(Weber *et al*., 2005). This work showed that RpoS affects the expression of approximately 10% (≈500 genes) of the *E. coli* genome under non optimal growth conditions, including stationary phase entry, acidification and hyperosmotic shift. For this reason, it has been referred to as 'the master regulator of the general stress response'. RpoS dependent genes can be grouped into 6 main categories: metabolism, regulation, transport and membrane, adaptation to stress, protein processing and those of an undefined function (Weber *et al.,* 2005).

RpoS can induce gene expression in two ways (reviewed by Battesti *et al*., 2011). Firstly, many RpoS-dependent genes simply show increased transcription in proportion to the amount of RpoS (and therefore RNAP bound to RpoS) in the cell. Weber *et al*. (2005) described a core group of 140 genes which were upregulated under all 3 stress conditions used (stationary phase entry, acidification and hyperosmotic shift), which strongly suggested that increased cellular levels of RpoS are sufficient to increase the levels of expression of these genes.

The remaining (approximately 350) genes required a specific stress in order to show induction. This implies that some RpoS controlled genes display more specific regulation, and require additional effectors to increase transcription. An example of a gene modulated in this way is *csiD*, which requires CRP and cAMP in addition to RpoS (Germer *et al*., 2001).

Somewhat paradoxically, some mutants with altered RpoS levels or activity have been shown to out-compete wild-type cells under some conditions. It seems these mutants are able to acquire nutrients more efficiently than wild-type cells, but that this comes at the cost of decreased stress resistance (Finkel, 2006). Despite this, RpoS mutants can be isolated at low levels from natural *E. coli* populations, and have been detected at approximately 2% in non-pathogenic *E. coli* populations (Snyder *et al*., 2012). However, it has also been shown that when RpoS mutants are grown in minimal medium, they display a much faster reduction in survival in long term stationary phase than their wild-type counterparts (Lange and Hengge-Aronis, 1991).

1.2.3 Control of RpoS levels

RpoS levels are under complex control by a number of mechanisms (summarised in Fig. 1.2). This is typical of alternative sigma factors, and means they are less likely to compete for RNAP when they are not required. The major mechanisms of regulation include transcriptional and translational control, degradation *via* proteolysis and modulation of RpoS activity (for recent review see Battesti *et al*., 2011). These work in concert to ensure that RpoS levels are low in unstressed, actively growing cells, but can quickly be increased on entry into stationary phase, when there is up to a 30 fold increase in RpoS levels (Hirsch and Elliot, 2005). RpoS is also upregulated in response to various stresses such as changes in osmotic pressure. However, for the purpose of this thesis, RpoS expression on entry into stationary phase will be the main focus here.

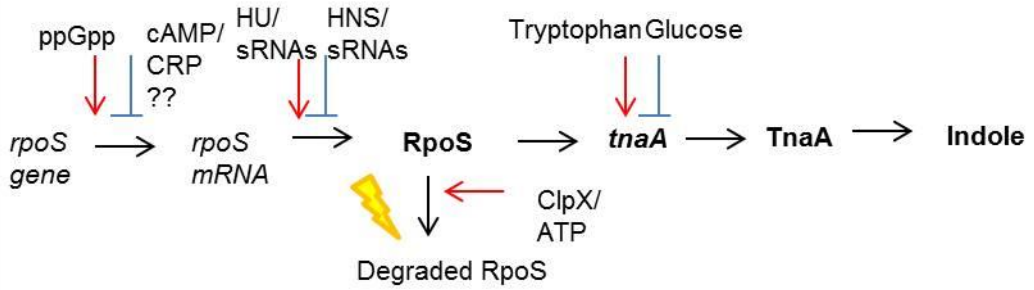


Figure 1.2 Diagram showing key players in control of RpoS levels, and down stream effects on tryptophanase and indole production. Red arrows indicate a stimulatory effect and blue arrows indicate an inhibitory effect.

1.2.3.1 Transcription of *rpoS*

The *rpoS* gene is located immediately downstream of *nlpD,* a gene encoding an outer membrane protein. Whilst the two relatively weak *nlpD* promoters control low level expression of *rpoS* in exponential phase, *rpoS* transcription is mainly driven by a single promoter (*rpoSp*1) located within the *nlpD* gene upstream of *rpoS* (Lange *et al*., 1995). This transcript originates 565 nucleotides upstream of the start codon for the *rpoS* gene, resulting in a long untranslated region (UTR) containing the ribosome binding site (RBS) which is key for translational control (Takayanagi *et al*., 1994).

In *E. coli* there is evidence for two important regulators of *rpoS* transcription. The *rpoS* promoter contains two putative cAMP/CRP binding sites, one upstream of the -35 sigma 70 consensus sequence and the other slightly downstream of the +1

transcription initiation position (Hengge-Aronis, 2002). Using an *rpoS::lacZ* transcriptional fusion reporter CRP/cAMP had been shown to negatively regulate transcription of *rpoS* in late exponential phase (Lange and Hengge-Aronis, 1994). This may seem counter-intuitive, as CRP/cAMP levels increase as glucose levels decrease, which might be expected at the onset to stationary phase. However, this had also been observed previously by Buettner *et al.* (1973), who showed no increase in transcription of *rpoS* at the onset of glucose starvation, where a burst of cAMP production occurs. Despite this Venturi (2003) referred to the role of cAMP/CRP in regulating *rpoS* transcription as 'controversial' and suggested that more work was required to verify whether the positions of the cAMP/CRP binding sites in the *rpoS* promoter are consistent with a role in negative regulation. Additionally, Hengge-Aronis referred to unpublished work suggesting that, during entry into stationary phase, cAMP-CRP positively controls *rpoS* transcription, indicating the mode of action of cAMP-CRP in *rpoS* transcriptional control may depend on the growth phase (Hengge-Aronis, 2002b).

In addition to the role of cAMP/CRP, the signalling molecule ppGpp also plays a key role in modulating RpoS expression. It has been shown that the expression of both transcriptional *rpoS::lacZ* fusions as well as the level of *rpoS* mRNA originating at rpoSp1 is strongly reduced in ppGpp-deficient *relA spoT* mutants (Lange *et al*., 1995). This suggests that ppGpp acts to positively regulate *rpoS* expression. ppGpp is used as an alarmone in the stringent response, so increasing rpoS expression in response to ppGpp seems plausible and intuitive, unlike the cAMP/CRP regulation discussed previously.

Despite these controls on *rpoS* transcription, they are not the major determinant of RpoS levels. More significant controls exist over translational and degradation (Lange and Hengge-Aronis 1994).

1.2.3.2 Translational control of *rpoS*

Global regulatory proteins contribute significantly to the translational regulation of RpoS. One is HU, a nucleoid associated, histone like protein. HU has been shown to bind with high affinity to a small *rpoS* mRNA fragment. It was also demonstrated that the efficiency of *rpoS* mRNA translation is reduced in mutant cells lacking

HU, which in turn suggested that HU stimulates *rpoS* translation directly during the onset to stationary phase (Balandina *et al*., 2001).

H-NS is another nucleoid associated protein which contributes to the regulation of *rpoS* translation*,* with H-NS mutants displaying a significantly increased amount of RpoS protein compared to wild-type cells. Furthermore proteins known to be under the control of RpoS were also shown to accumulate in a H-NS mutant strain (Barth *et al.,* 1995). However, it is still unclear how H-NS acts to decrease RpoS translation (Hengge-Aronis, 2002b).

Small RNAs (sRNAs) also play an important role in translational regulation of *rpoS*, by interacting with the UTR of the *rpoS* transcript. One example is DsrA which increases *rpoS* translation (Majdalani *et al*., 1998). Typically, in the absence of the sRNAs the UTR folds into a stem loop structure which occludes the ribosome binding site, thus inhibiting translation of *rpoS.* In the presence of the sRNAs and RNA chaperone Hfq, the inhibition of translation of *rpoS* is overcome and RpoS protein is produced. It is clear that *E. coli* uses a wide range of mechanism to control the levels of the RpoS protein on entry into stationary phase.

1.2.3.3 RpoS degradation and modulation of activity

In exponentially growing cells RpoS is produced but is subject to rapid degradation, whereas in non-optimal conditions such as entry into stationary phase, degradation becomes slower allowing RpoS to accumulate.

RpoS degradation is performed by the AAA+ ATPase ClpXP proteosome. This process is facilitated by the response regulator RssB (also known as SprE), which directs RpoS to the protease (Muffler *et al.,* 1996). Recent work has highlighted the importance of ATP in RpoS proteolysis. In an *in vitro* assay ClpXP failed to specifically degrade RpoS in the presence of low ATP, implying that the level of ATP in the cell contributes directly to RpoS proteolysis (Peterson *et al*., 2012). The outcome of all these controls is that RpoS accumulates on entry into stationary phase.

1.3 Indole as a signalling molecule

Indole is an aromatic heterocyclic molecule. It is formed of a five membered ring, which contains a nitrogen atom, attached to a six membered benzene ring, as shown in Fig. 1.3 (Roychowdhury and Basak, 1975).

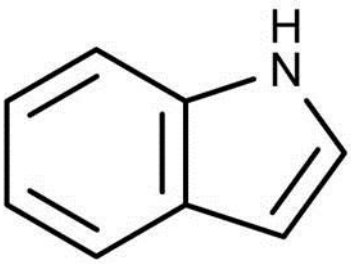


Figure 1.3 Chemical structure of indole

Indole is produced by tryptophanase (TnaA) which is encoded by the *tnaA* gene. Tryptophanase catalyses the conversion of tryptophan to indole, pyruvate and ammonia (Newton and Snell, 1965). *E. coli* tryptophanase functions as a tetramer of four 52.8 kDa subunits , and the activity of tryptophanase is dependent on pyridoxal 5'-phosphate (PLP, vitamin B_6) which binds to a lysine residue at position 270 (Ku *et al.*, 2006). Whilst the reaction is reversible, it is heavily thermodynamically biased to produce indole (Tewari and Goldberg, 1994). Furthermore, it is interesting to note that no specific enzyme exists in *E. coli* to degrade indole (Chant and Summers, 2007). As a results of this, when indole is made by tryptophanase, it diffuses out of the cell, and accumulates in the supernatant where it remains. This is completely different from all the nucleotide signals mentioned previously in the introduction, which can be actively hydrolysed. A wide variety of bacteria encode the tryptophanase (*tnaA*) enzyme and therefore can produce indole. Lee and Lee (2010) listed over 85 indole-producing species in a recent review. These include Gram-negative, Gram-positive, pathogenic and non-pathogenic strains.

However, Zhang and Hong (2009) suggested that making indole may actually confer a disadvantage to *E. coli.* They compared the optical density of BW25113 and BW25113 Δ*tnaA* (an isogenic indole non-producing mutant with tryptophanase knocked out) growing in LB medium at 37°C, with shaking, through exponential phase and into stationary phase. They showed that the BW25113 Δ*tnaA* cells entered stationary phase with a significantly higher cell density than the wild-type

cells, suggesting a growth advantage is conferred when no indole is produced. This is an interesting observation, which will be subject to further study and analysis in Chapter 3.

It has recently been shown that indole is able to pass freely across the cell membrane. Historic reports proposed that indole was able to diffuse across cell membranes (Bean *et al*., 1968) but in more recent years, it had been widely claimed that protein transporters were required for indole to get in and out of the cell. The major import protein for indole was reported to be Mtr. This conclusion was reached using an experimental system where an *E. coli* mutant strain, deficient in the tryptophan biosynthetic pathway, could be rescued on tryptophan-free medium by the addition of exogenous indole. Presumably, the indole was taken up and converted to tryptophan, by tryptophanase, inside the cell. This could not be done in a strain also containing an *mtr* mutation, which implied that indole was taken up by the Mtr protein (Yanofsky *et al*., 1991).

The protein responsible for indole export was widely assumed to be the AcrEF multidrug exporter. This conclusion was based upon a study which showed that indole accumulation in the culture supernatant of an *E. coli* AcrEF deletion strain was reduced, whilst the intracellular indole concentration was increased (Kawamura-Sato *et al.,* 1999).

However Piñero-Fernandez *et al.* (2007) provided evidence that indole is able to pass directly through the *E. coli* cell membrane without the assistance of protein transporters. Experiments utilized an intracellular indole sensor, the pStyABB plasmid which encoded styrene monooxygenases which convert intracellular indole into indigo, which can be detected spectroscopically. When indole was added to the culture supernatant, there was no significant difference in the rate of accumulation of indigo, and therefore intracellular indole, in the cells in the presence of absence of Mtr. In addition to this Piñero-Fernandez *et al.* (2007) compared the sensitivity of cells to indole with and without the AcrEF multidrug exporter. If AcrEF exports indole, the mutant cells should display higher sensitivity to indole due to an elevated intracellular indole concentration. This was shown not to be the case, which suggested that the AcrEF multidrug exporter is not important in indole export. Finally, work performed using liposomes made from *E. coli* total lipid extract allowed

direct observation of indole transport across cell membrane without the aid of protein transporters (Piñero-Fernandez *et al.,* 2007).

These observations suggest that as *E .coli* makes indole it diffuses out of the cell into the surrounding environment. This means that in an environment such as the human gut even indole non-producing bacteria are likely to come into contact with indole. As no specific proteins are required for indole uptake, even indole non-producing bacteria will be exposed to the indole as it passes across the cell membrane. This means that indole can have effects on species of bacteria which are unable to produce it themselves.

1.3.1 The effects of indole on *E. coli*

Low levels of indole (0.5- 1 mM) are used as a signal to modulate many processes in *E. coli.* Indole has been shown to have a wide variety of effects on gene expression including the induction of a range of xenobiotic exporter genes including *acrD*, *acrE*, *cusB* and *emrK* (Hirakawa *et al*., 2005). This correlated with a 2.8 fold increase in survival of cells when exposed to carbenicillin. This suggests that indole may contribute to antibiotic resistance to *E. coli.*

The expression of other genes including *gabT*, *astD* and *tnaB* is also modulated in the presence of indole (Wang *et al*., 2001). These effects of indole on gene expression have led some reports to refer to indole as a quorum sensing molecule (Lee *et al*., 2009, Walters and Sperandio, 2006). However, in some cases, chemical complementation of indole non-producing mutant cells with external indole and/or the classical ‘spent medium’ experiment are unable to recapitulate the phenotype of wild-type cells. Furthermore, there is currently no data on the mechanism by which indole acts to alter gene expression *via* a receptor or otherwise. For these reasons, whether or not indole is a quorum sensing molecule is very much still a matter of debate in the literature (Winzer *et al.,* 2002). This point will be revisited in the discussion, where the results described in this thesis will contribute to this debate.

Indole signalling is also important in controlling the virulence of pathogenic *E. coli*. Enterohaemorrhagic *E. coli* (EHEC) O157:H7 is a pathogenic *E. coli* strain which can cause severe infections in humans. In order to do this it must adhere to the human gut cells, this process triggers the formation of hallmark lesions called attaching and effacing (A/E) lesions (Nataro and Kaper, 1998). The ability to form A/E lesions is conferred by a type III secretion system and the proteins secreted by it. These proteins are gene products of the locus of enterocyte effacement (LEE), a chromosomal pathogenicity island containing five major operons (LEE1 to LEE5) (McDaniel and Kaper, 1997). Hirakawa *et al.* (2009) showed that indole increased the secretion and production of EspA/B (a LEE4 gene product). Additionally, indole increased production of type III-dependent A/E lesions in EHEC.

Furthermore, it was shown the tryptophanase gene is required in order for pathogenic *E. coli* to cause paralysis and host killing in the *Caenorhabditis elegans* model (Anyanful *et al*., 2005). Here, supplementation of a non indole producing mutant with external indole at a range of concentrations was insufficient to restore the wild-type phenotype. EHEC required indole production alongside the chemically related compounds indole-3-carboxaldehyde (ICA) and indole-3-acetic acid (IAA), which are also made by EHEC, and by commensal *E. coli* to act in synergy to kill *C. elegans* (Bommarius *et al*., 2013).

Indole has also recently been implicated in the formation of *E. coli* persisters. These are a sub-population of cells which are able to tolerate and survive antibiotic treatment, by slowing growth and becoming dormant (Gefen and Balaban, 2009). Persisters typically form at the transition into stationary phase, which is when indole is produced. Vega *et al.* (2013) showed that in indole non-producing mutants, persister formation was decreased by approximately an order of magnitude and that this could be restored to wild- type levels by addition of 0.5 mM indole. Microarray analysis indicated that addition of indole significantly increased expression of genes in the OxyR and phage-shock pathways. The authors suggested that indole is able to ‘inoculate’ a population by activating these stress responses, which contribute to bacterial stasis and persistence.

Additionally, low levels of indole have been shown to have various effects on *E. coli* biofilm formation. DiMartino *et al.* (2003) demonstrated that in *E. coli* S17-1 indole

increased biofilm formation. However in subsequent studies, indole was shown to reduce biofilm formation of nine other non-pathogenic *E. coli* strains (Domka *et al*., 2006; Lee *et al*., 2007).

Indole also increases the survival of *E. coli* exposed to acidic conditions (Hirakawa *et al*., 2010). The authors showed that the addition of 1-2 mM indole to *E. coli* increased the expression of acid resistance genes of the glutamine decarboxylase system, which in turn increased acid resistance.

It is clear that there is a large, and at times, confusing and contradictory literature regarding the effect of low levels of indole of *E. coli* physiology.

1.3.2 Indole as a bacterial inter-species signal

Indole has been shown to affect biofilm formation in various other species of bacteria. Di Martino *et al.* (2003) used a chemical inhibitor of tryptophanase, oxindolyl-L-alanine, to inhibit indole production in clinical strains of bacteria. Reduced indole production in species including *Klebsiella oxytoca, Citrobacter koseri, Providencia stuartii*, and *Morganella morganii* resulted in reduced biofilm formation. Biofilm formation in *V. cholerae* is also affected by the presence of indole. Environmental isolates of *V. cholerae* which form significantly reduced biofilms were shown to have a transposon insertion in the tryptophanase gene. Exogenous indole (0.35 mM) was added to these mutant strains, and biofilm formation was restored to the level observed in the wild-type strain, suggesting that indole is a key player in biofilm formation in *V. cholerae* (Mueller *et al.,* 2007). In a follow up study, indole was shown to induce expression of the *vspL* gene, which is involved in vibrio polysaccharide biosynthesis, and is essential for biofilm formation. In addition, microarray analysis demonstrated that indole altered the expression of various other genes involved in ion transport and motility (Mueller *et al.,* 2009).

Salmonella typhimurium does not encode tryptophanase and therefore cannot make indole. However it is likely to come into contact with indole made by *E. coli* in an environment such as the human gut. It has been shown that addition of indole to *S. typhimurium* resulted in a significant increase in the expression of genes encoding the multidrug exporters *acrAB, emrAB, acrD*, and *mdtABC.* Furthermore, this correlated with an increased survival when *S. typhimurium* was exposed to

benzalkonium, suggesting that indole enhanced drug tolerance of *S. typhimurium* (Nikaido *et al*., 2008).
In a follow-up study Nikado *et al.* (2012) demonstrated that in the presence of 1 and 4 mM indole, 24 *S. typhimurium* genes were upregulated, including *ramA, ydiP, yhjB, and bglJ*. These genes all have regulatory functions, and their expression was shown to be increased at least 10 fold in the presence of indole. Additionally, 53 *S. typhimurium* genes were down regulated, these included genes involved in flagella biosynthesis (*flgN/K/L* and *fliD/S/T*) and chemotaxis (*cheB/R/M/ tcp*, and *trg*). They also showed that motility and invasion of *S. typhimurium* were reduced in a phenotypic assay in the presence of indole. The authors speculated that indole made by *E. coli* in the human gut reduces the virulence of *Salmonella*, which may provide an advantage to *E. coli*.

In a study by Vega *et al.* (2013) it was shown that *S. typhimurium* has increased antibiotic tolerance in the presence of indole. When exposed to carbenicillin, the addition of 0.5 mM indole to *S. typhimurium* cultures increased survival three-fold. Interestingly, a three-fold increase in survival on exposure to ciprofloxacin required a higher concentration (1.25 mM) of indole. The authors suggested that *S. typhimurium* may potentially benefit from the presence of indole in this way.

Another bacterium which does not encode tryptophanase, so cannot make indole, yet is affected by the presence of indole, is the model soil bacterium *Pseudomonas putida.* Addition of 2 mM indole to cultures of *P. putida* significantly reduced the growth rate, with concentrations in excess of 3 mM inhibiting growth almost completely. Furthermore, in the presence of 2-3 mM indole the ATP levels were significantly reduced and the NADH/NAD+ ratio significantly increased. Expression of 47 genes in *P. putida* was also shown to be altered in the presence of indole. Of these, 23 were either involved in the tricarboxylic acid cycle, or had functions as chaperones or proteases. (Kim *et al*., 2013).

1.3.3 Indole as an inter-kingdom signal

Indole is also able to act as an interkingdom signal. In an environment such as the human gut, which contains indole producing bacteria, the surroundings are exposed to indole as it diffuses out of the producer cells. Indole has been shown to affect

gene expression in human gut cells. In the presence of 1 mM indole the human HCT-8 intestinal epithelial cell line showed increased expression of over 500 genes, including those encoding claudin and tight junction proteins, which play key roles in strengthening the mucosal barrier. This was consistent with an increase in the transepithelial resistance of HCT-8 cells. In addition, expression of inflammatory cytokine and chemokines were increased. The authors speculated that indole may serve as a beneficial interkingdom signal that helps to reinforce the mucosal barrier and therefore potentially increase resistance to pathogen colonisation (Bansal *et al*., 2010).

The role of indole in modulating the behaviour of eukaryotic cells *in vivo* was confirmed recently. Shimada *et al*. (2013) investigated the effect of indole on gut epithelial cells using a germ free (GF) mouse model. They showed that GF mice had decreased expression of the tight junction proteins compared to the wild-type controls, and that this differential expression could be restored by feeding the mice indole containing capsules. The authors conclude that their findings confirmed the beneficial role of indole, made by commensal bacteria, in establishing an epithelial barrier *in vivo*.

Whilst these are all relatively recent examples of indole influencing eukaryotic cells, around 30 years ago a report was published showing the effect of indole on oxidative phosphorylation in rat liver mitochondria. When rat livers were perfused with indole (0.5- 5 mM) various effects were observed including a reduction in the adenylate energy charge. Furthermore, some effects were concentration dependent. For example with 0.5 mM indole the oxygen concentration in the effluent fluid was decreased but in the presence of 5 mM indole it was increased (Sakai *et al*., 1982).
Since then, further work has demonstrated that addition of indole to isolated rat liver mitochondria increases the rate of mitochondrial oxygen consumption significantly Again, this effect showed concentration dependence, at concentrations of indole below 0.4 mM the oxygen consumption rate was unaffected, but when the concentration of indole was increased from 0.4 mM to 0.8 mM, the oxygen consumption rate increased by 60% (Chimerel *et al.,* 2013). Clearly, indole has diverse roles in both bacterial and host physiology, many of which have recently become apparent.

1.3.2 Production of indole in *E. coli*

The work described in this thesis will concentrate solely on the production and effects of indole in *E. coli*. As far back as 1889, indole production was used as a diagnostic test to distinguish *E. coli* from *Klebsiella* species (Isenberg and Sundheim, 1958). Indole production can be assayed by a variety of methods, the most widely used being Kovacs assay (Kovac, 1928).

1.3.2.1 Control of tryptophanase levels

Tryptophanase expression is under a series of stringent controls in *E. coli,* including catabolite repression and transcription attenuation mechanisms, which have been well studied by the Yanofsky group and others for over 30 years (for recent review, Yanofsky, 2007). Tryptophanase *(tnaA)* forms part of the tryptophanase *(tna)* operon (Fig. 1.4), along with another major structural gene *tnaB. tnaB* encodes a tryptophan specific permease which imports tryptophan into the cell (Deeley and Yanofsky, 1981, Edwards and Yudkin, 1982). *In vitro* transcription experiments revealed that the transcription initiation site was located 320 base pairs upstream of *tnaA* (Deeley and Yanofsky, 1982). Also encoded in the region upstream of *tnaA* is a 24 bp region encoding a leader peptide, called *tnaC* (Stewart and Yanofsky, 1985). Downstream of *tnaC* there is a non-coding region which contains several Rho-dependent terminator sequences.

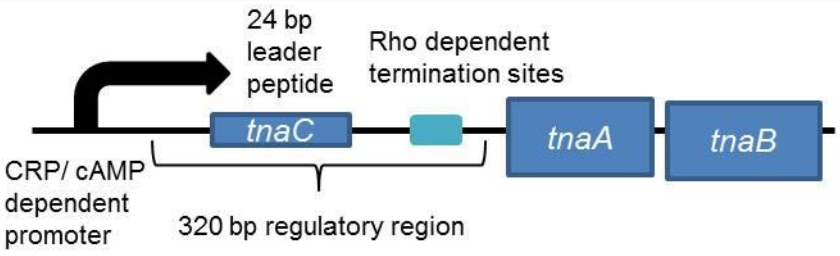


Figure 1.4 The *tna* operon of *E. coli*. The *tna* operon consists of a CRP/cAMP dependent promoter, a 320 bp regulatory region which includes *tnaC* and Rho dependent termination sites and genes encoding tryptophanase (*tnaA*) and a tryptophan specific permease (*tnaB*). Note: not drawn to scale

1.3.2.1.1 Tryptophanase expression is subject to catabolite repression

Initiation of transcription of the *tna* operon is controlled by catabolite repression (Botsford and Demoss, 1971). This means that initiation of transcription requires cAMP-dependent, CRP-facilitated binding of RNA polymerase to the *tna* promotor (Ward and Yudkin, 1976, Deeley and Yanofksy, 1982). At low glucose concentrations, cAMP accumulates and binds to CRP which in turn binds to the *tna* promoter, recruiting RNA polymerase to initiate transcription. Conversely, at high glucose concentrations, cAMP accumulation is inhibited, CRP is unable to bind to the *tna* promoter and transcription of the *tna* operon is repressed. The outcome is that glucose levels must be depleted for transcription of the *tna* operon to occur.

This is presumably one of the reasons why significant levels of indole are only detected in culture supernatants when the cells are approaching stationary phase (Chant and Summers, 2007). Furthermore, addition of glucose to *E. coli* cultures can be used to delay indole production (Bansal *et al*., 2007).

1.3.2.1.2 Tryptophanase expression is regulated by transcription anti-termination.

It has been known for some time that induction of the *tna* operon is tryptophan dose-dependent up to 100 μg ml^{-1} (Yanofsky *et al*., 1991). The extension of the *tna* operon transcripts is regulated by transcription anti-termination. In the absence of the inducer, tryptophan, transcription is terminated prematurely at the Rho-dependent termination sequences (Stewart and Yanofsky, 1985). In the presence of tryptophan, termination is prevented, the *tna* transcript is not attenuated at the Rho sites and mRNA containing *tnaA* and *tnaB* is produced (Stewart and Yanofsky, 1985).

When tryptophan is absent, the ribosome cleaves the TnaC-peptidyl-$tRNA^{Pro}$ and dissociates from the mRNA upon recognition of *tnaC* stop codon. Release of the ribosome exposes the Rho factor binding sites immediately downstream of the *tnaC* stop codon. The Rho factor consequently binds and interacts with the paused RNA polymerase and acts to terminate transcription. Conversely, in the presence of high concentrations of tryptophan, tryptophan binds near the ribosomal A site (Cruz-Vera *et al*., 2006), blocking the peptidyl transferase activity. The critical tryptophan recognition site is created by both the TnaC nascent peptide and 23S rRNA of the ribosome. As a result of tryptophan binding, the ribosome cannot cleave the TnaC-

peptidyl-tRNA^{Pro} nor dissociate from the nascent pepetide. The stalled ribosome prevents the Rho factor from recognising and binding to the Rho factor binding sites in the leader region and consequently, transcription of *tnaA* and *tnaB* occurs (Cruz-Vera *et al*., 2007).

There is a further level of control of the tryptophanase operon. Lacour and Landini (2004) showed that tryptophanase is under the control of RpoS, the stationary phase sigma factor. RpoS itself is under the control of a series of precise controls, reviewed in section 1.2.3. In an RpoS null mutant tryptophanase mRNA levels are reduced approximately 14 fold compared to a wild-type strain. They also showed that this correlated with a significantly reduced level of indole production in the RpoS mutant. The outcome of these 3 controls is that a high level of tryptophanase expression is not possible until RpoS levels have increased during the entry to stationary phase, and until glucose levels have been depleted and tryptophan levels are high.

1.3.5 Environmental factors affecting indole production in *E. coli*

The experiments described above refer to *E. coli* producing indole under standard laboratory conditions, i.e. growth in LB medium, at 37 ° C with shaking. Under these conditions indole production occurs during stationary phase. Levels of indole detected in the supernatant at this time can vary slightly depending on the measurement technique and the specific *E. coli* strain, but typically fall into a range of between 0.5-1 mM (Lee *et al*., 2007, Chant and Summers, 2007, Han *et al*., 2011, Li and Young, 2013). This corresponds well to the concentration of free tryptophan in LB medium, which has been estimated at between 0.5 mM (Li and Young, 2013) and 1 mM (Sezonov *et al*., 2007). This suggests that under standard conditions *E. coli* will use tryptophanase to convert all available tryptophan into indole, during stationary phase entry. This was shown to be true by Li and Young (2013) who showed that addition of tryptophan to *E. coli* growing in minimal media results in equal conversion to indole, up to a concentration of 5 mM. The effect here is twofold, tryptophan acts to induce further tryptophanase expression, and the additional tryptophan simply provides more substrate for tryptophanase to stoichiometrically convert into indole.

However, there are reports in the literature which show that indole production can be affected by external factors including stresses such as changes in temperature and pH or exposure to antibiotics and oxidative stress.

The temperature at which cells are grown at can affect indole production. Han *et al.* (2011) reported that when *E. coli* BW25113 was grown at 25 °C and 37 °C, indole accumulated to approximately 0.5 mM during stationary phase, which is consistent with the reports of others. However, when cells were incubated at 50°C, very little cell growth was observed but indole accumulated quickly to approximately 0.5 mM, suggesting each cell was producing significantly more indole at this higher temperature.

Han *et al.* (2011) also assessed the effect of pH on indole production in *E. coli* BW25113 at both 30°C and 37°C. They reported that low pH inhibited indole production whilst high pH increased indole production both at 30 °C and at 37 °C. This result correlated well with previous work performed by Maurer *et al.* (2005) who showed that at pH 5 *tnaA* expression was reduced, whilst at pH 9 *tnaA* expression was increased.

When Han *et al.* (2011) investigated indole production in the presence of various antibiotics, they showed that indole production per cell was increased in the presence of both ampicillin and kanamycin. It has also been shown that under conditions of oxidative stress (elevated H_2O_2 and superoxide ion levels) due to mutations or certain antibiotics (Kuczyńska-Wiśnik *et al*., 2010a), tryptophanase activity is up-regulated and the extracellular indole concentration increases accordingly (Kuczyńska-Wiśnik *et al*., 2010b).

It is tempting to suggest that there is a single mechanism behind all of the above affects. Changes in temperature and pH, and exposure to antibiotics and oxidative stress are likely to cause RpoS levels to increase (Battesti *et al*., 2011). This in turn will induce tryptophanase expression, allowing increased indole production. This would be further evidence that indole is an important signalling molecule in helping cells respond to a variety of stresses.

1.3.6 The roles of high levels of indole in *E. coli*

The effects of indole on *E. coli* physiology are wide and varied, and also show concentration dependence. Until recently, only the effects of low, persistent concentrations (0.5- 1 mM) of indole on *E. coli* have been studied in detail as these are believed to be physiologically relevant as they are detected in culture

supernatants (reviewed recently by Lee and Lee, 2010). However, it is becoming apparent, both in recently published work and throughout this thesis that high, transient levels of indole can also have important effects in *E. coli.*

Recently, the effects of indole at higher levels (3-5 mM) have begun to be elucidated in our laboratory. These investigations came out of the study of multicopy plasmid stability, as shown in Fig. 1.5. In the absence of active partitioning, high copy number plasmids can be stably maintained by their host cells provided that copy number remains high. Plasmid dimerization, the process by which two plasmids monomers undergo homologous recombination to form a single plasmid dimer, acts to reduce the effective copy number in a cell (Summers and Sherratt, 1984). Furthermore, two origins of replication are present in a plasmid dimer, hence a dimer replicates twice as often as a monomer, causing dimers to accumulate rapidly in the descendants of the cell in which it originally arose. Together these processes make up what has been coined the dimer catastrophe (Summers *et al.,* 1993). Recent reanalysis of the dimer catastrophe hypothesis suggests that plasmid dimers pose a threat to the host for a different reason, the cells containing dimers experience a significant metabolic penalty. This decreases the fitness of the dimer containing host, compared to its monomer containing counterparts (Field and Summers, 2011). It has been suggested that plasmid dimerization can be viewed as a form of DNA damage, a stress which the cell must overcome.

In response to the threat posed by the dimer catastrophe, plasmids employ site-specific recombination to resolve plasmid multimers to monomers. A well-studied example is the Xer-*cer* system employed by plasmid ColE1 (Summers and Sherratt, 1984, Colloms *et al.*, 1990, Blakely *et al.*, 1993, Stirling *et al.*, 1988 and 1989). Although the Xer-*cer* recombination system is able to convert plasmid dimers to monomers, timing is also crucial. This conversion must be completed before the cell divides, since this is when the plasmid could potentially be lost.

A promoter, P_{cer}, encoded within the *cer* site (Summers and Sherratt, 1988) controls transcription of a short regulatory RNA known as Rcd (regulator of cell division). It was observed that increased levels of Rcd were present in dimer containing cells and that over-expression of Rcd delays cell division (Patient and Summers, 1993). This led to the development of the hypothesis that Rcd is part of a checkpoint that

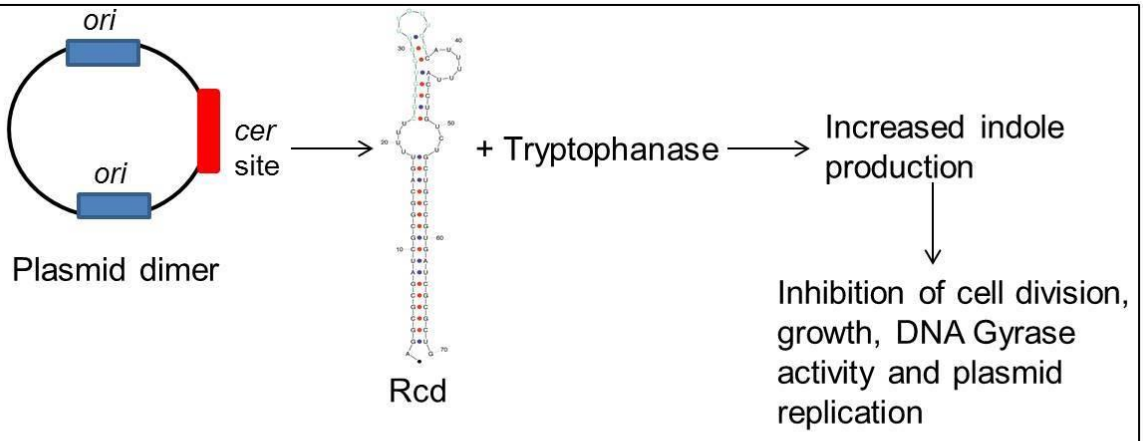


Figure 1.5 The role of indole in the dimer catastrophe. Plasmid dimers express Rcd from the *cer* site. Rcd binds to tryptophanase, increasing its affinity for tryptophan. This in turn allows increased indole production, which inhibits cell division, growth, DNA Gyrase activity and plasmid replication.

prevents cell division prior to dimer resolution. In order to identify the target of Rcd, RNA affinity chromatography was performed using crude cell lysates and identified tryptophanase as an Rcd binding protein (Chant and Summers, 2007). In the presence of Rcd the affinity of tryptophanase for tryptophan is increased by approximately 5 fold, which in turn increases the levels of pyruvate, ammonia and indole in the cell. It was shown that addition of between 3-5 mM indole significantly inhibits division of cells in broth culture, suggesting that an increased intracellular indole concentration is the mechanism by which Rcd delays cell division (Chant and Summers, 2007).

Reanalysis of the dimer catastrophe theory suggested that simply delaying cell division is insufficient to contain the dimer catastrophe, as the dimers would continue to out-replicate monomers in the non-dividing cell (Field and Summers, 2011). This led to the hypothesis that indole might also inhibit plasmid replication. It appears that this is achieved by inhibiting the action of DNA gyrase, as addition of 3-5 mM of indole inhibits DNA gyrase *in vitro* and plasmid replication *in vivo* (Field and Summers, 2012).

The mechanism by which indole inhibits *E. coli* cell division has recently been elucidated. At high concentrations (3-5 mM) indole acts as an ionophore to disrupt the proton gradient across the cytoplasmic membrane and reversibly inhibit cell division (Chimerel *et al*., 2012). The loss of the proton gradient across the cell membrane prevents MinD oscillation, which disrupts the proper localisation of FtsZ, inhibiting cell division. This was the first example of a natural ionophore acting to

regulate an important biological process. Furthermore, the idea that indole could be targeting the cell membrane directly offered a novel way for indole to affect *E. coli* physiology.

However, a potential criticism of the work of Chimerel *et al*. (2012) was that the inhibition of *E. coli* cell division required the addition of 3-5 mM indole to the culture supernatant. This is approximately 10 times the concentration of indole which is detected in culture supernatants of *E. coli* grown in LB medium under standard laboratory conditions. As such the biological relevance of the effects of these higher indole concentrations has been questioned.

1.4 Aims of this work

The aim of this work was to assess the physiological relevance of high indole concentrations. The most important question to ask was whether *E. coli* cells ever experience high transient levels of indole. If so, are these levels sufficient to inhibit cell division and growth?

One way to achieve significantly higher levels of indole could be through an increased production rate. Therefore, the production rate of indole by *E. coli* will be measured throughout different growth phases. The role of indole production at the onset of stationary phase will also be examined in detail. A method to measure 'cell-associated' indole will be developed and these assays performed. The mechanisms by which indole production can be upregulated by *E. coli* will also be verified, the role of RpoS, tryptophanase, tryptophan and glucose will be studied in detail.

Chapter 2

Materials and Methods

2.1 Strains

Table 2.1: List of *E. coli* strains used in this work

Name of Strain	Genotype/ Description	Source/Reference
BW25113	*lacI^q rrnB$_{T14}$* Δ*lacZ$_{WJ16}$ hsdR514* Δ*araBAD$_{AH33}$* Δ*rhaBAD$_{LD78}$*	Laboratory stock Datsenko and Wanner, 2007
BW25113 Δ*tnaA*:: Km^R	BW25113 Δ*tnaA*:: Km^R Tryptophanase knockout, Kanamycin resistant.	Laboratory stock Datsenko and Wanner, 2007
BW25113 Δ*rpoS*:: Km^R	BW25113 Δ*rpoS*:: Km^R RpoS knockout Kanamycin resistant	Kindly supplied by: *E. coli* Genetic Stock Center (CGSC) http://cgsc.biology.yale.edu/index.php
BW25113 Δ*rpoS*: Km^S	*lacI^q rrnB$_{T14}$* ΔlacZ$_{WJ16}$ hsdR514 ΔaraBAD$_{AH33}$ ΔrhaBAD$_{LD78}$ RpoS knockout Kanamycin sensitive, Km^R gene removed using pCP20 plasmid	This work
MG1655	F$^-$ λ$^-$ *ilvG$^-$ rfb-50 rph-1*	Kindly supplied by Gang Li and Kevin D. Young Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199, USA.

MG1655 *TnaA–GFP*	MG1655 *tnaA–GFP:: frt*	Li and Young (2012)
MG1655 *TnaA–GFP :: Km^R*	MG1655 *tnaA–GFP* Kanamycin resistance	Li and Young (2012)
BW25113 Δ*rpoS TnaA–GFP:: Km^R*	*$lacI^q$ $rrnB_{T14}$* Δ$lacZ_{WJ16}$ *hsdR514* Δ*$araBAD_{AH33}$* Δ*$rhaBAD_{LD78}$* Δ*rpoS TnaA–GFP: Km^R* RpoS knockout *tnaA–GFP:* Km^R introduced from MG1655 *TnaA–GFP : Km^R* by P1 transduction Kanamycin resistant	This work
BW25113 *TnaA–GFP: Km^R*	*$lacI^q$ $rrnB_{T14}$* Δ*$lacZ_{WJ16}$ hsdR514* Δ*$araBAD_{AH33}$* Δ*$rhaBAD_{LD78}$* *tnaA–GFP*: Km^R introduced from MG1655 *tnaA–GFP* by P1 transduction Kanamycin resistant	This work

Table 2.1 continued: List of *E.coli* strains used in this work

2.2 Plasmids

Table 2.2: List of plasmids used in this work

Name of Plasmid	Genotype/ Description	Source/Reference
pStyAAB	Constitutively expresses *styA* and *styB* (styrene monooxygenases) from *Pseudomonas putida.* Used to convert indole into indigo *in vivo* Carbenicillin resistant.	O'Connor *et al.*, 1997
pCP20	Temperature-sensitive replication Thermal induction of FLP synthesis Used to remove Kanamycin resistance gene from knockout strains Carbenicillin resistant Chloramphenicol resistant.	Cherepanov and Wackernagel,1995

2.3 Media

2.3.1 Luria Broth Base (LB)

Supplied by ForMedium™

Solution was autoclaved at 121°C for 20 minutes before use.

Component	**Concentration/ gL^{-1}**
Tryptone	10
Yeast Extract	5
NaCl	0.5

Table 2.3: Components of LB medium

2.3.2 Luria Broth Base with Agar (LA)/ Soft agar (SA)

For LA 1.5% agar was added to LB medium

For SA 0.7% agar was added to LB medium

Solution was autoclaved at 121°C for 20 minutes before use.

2.3.3 Minimal Medium

Component	**Final concentration**
M9 salts	X1 (as stock from Sigma)
Glucose	0.2%
$CaCl_2$	0.1 mM
$MgSO_4$	2 mM

Table 2.4: Components of minimal medium

All components were filter sterilized using a 0.2 µm filter before use and stored at 4°C.

Appropriate volumes of minimal medium without glucose were made using distilled water and autoclaved at 121°C for 20 minutes prior to use. Glucose was added subsequently.

2.4 Antibiotics

Antibiotic	Stock concentration and solvent	Working concentration	Supplier
Carbenicillin	100 mg ml^{-1} in distilled water	100 µg ml^{-1}	Melford
Kanamycin	30 mg ml^{-1} in distilled water	30 µg ml^{-1}	Melford
Chloramphenicol	34 mg ml^{-1} in ethanol	34 µg ml^{-1}	Melford

Table 2.5: List of antibiotics used in this work

All antibiotics were filter sterilized using a 0.2 µm filter before use, aliquoted and stored at -20 °C.

2.5 Chemicals

Chemical	**Stock concentration and solvent**	**Supplier**
Indole	50 mM in ethanol	Sigma
Tryptophan	20 mM in distilled water	Sigma
$CaCl_2$	1 M in distilled water	Sigma
Sodium Citrate	0.5% (w/v) in distilled water	Sigma

Table 2.6: List of chemicals used in this work

Indole was protected from light by wrapping in aluminium foil and storing at 4°C.

All other chemicals were filter sterilized using a 0.2 µm filter before use, and stored at 4 °C

2.6 Microbiological Techniques

2.6.1 Stock maintenance

For short term storage, strains were streaked to single colonies on LA plates with appropriate antibiotics. Strains were left to grow overnight (~16 hours) at 37°C, or 30°C for temperature sensitive strains, before sealing with Parafilm and storing at 4°C.

For long term storage, 1.35 ml of an overnight culture was mixed with 0.45 ml sterile 60% glycerol in a 2 ml cryovial. This was frozen in liquid nitrogen and stored at -80°C.

2.6.2 Growth curves

Appropriate medium (10 ml), with appropriate antibiotic if required, was inoculated with a single *E. coli* colony and incubated overnight with shaking at 120 rpm at 37 ºC, or 30 ºC for temperature sensitive strains. Overnight cultures were diluted to an OD_{600} of 0.05 in appropriate medium and incubated with shaking at 120 rpm at 37 ºC, or 30 ºC for temperature sensitive strains. Growth was measured by monitoring optical density at 600 nm (OD_{600}) using a Gene Quant 1300, GE Spectrophotometer.

2.6.3 Measuring colony forming units (CFU)

Appropriate medium (10 ml), with appropriate antibiotic if required, was inoculated with a single *E. coli* colony and incubated overnight with shaking at 120 rpm at 37 ºC, or 30 ºC for temperature sensitive strains. Overnight cultures were diluted 1:100 in appropriate medium and incubated with shaking at 120 rpm at 37 ºC, or 30 ºC for temperature sensitive strains. Samples were removed from the culture, diluted appropriately and spread onto LA plates, containing appropriate antibiotics. The plates were incubated at 37 ° C overnight, and colonies counted the next day.

2.6.4 Electroporation

LB medium (10 ml) was inoculated with a single *E. coli* colony and incubated overnight with shaking at 120 rpm at 37ºC, or 30ºC for temperature sensitive strains. Cells were harvested by centrifugation (10 minutes, 2880 x g at 4 ºC Eppendorf 5810R Centrifuge). The supernatant was discarded and the pellet resuspended in 10 ml of ice cold sterile distilled water. This was repeated three times, the final pellet was resuspended in 50 µl of ice cold sterile distilled water. This was transferred to an electroporation cuvette, 1 µl of plasmid DNA stock was added and mixed well. The mixture was subjected a single pulse of 1.68 kV through a 200-25 µF resistor-capacitor pair with a Gene Pulser electroporator (Biorad). 500 µl of LB medium was added and the mixture transferred to a 1 ml tube. The mixture was left for 1 hour with shaking at 120 rpm at 37ºC, or 30ºC for temperature sensitive strains. Appropriate dilutions of transformed cells were spread onto LA plates containing appropriate antibiotics.

2.6.5 P1 Transduction

P1 transduction allows portions of the *E. coli* chromosome to be moved from one strain into another. In this work it was used to transfer *TnaA:GFP* Km^R from MG1655 *TnaA:GFP* Km^R into BW25113 and BW25113 Δ *rpoS*.

The experimental procedure consists of two parts.

2.6.5.1 Preparation of donor lysate

A sample of overnight culture (500 ml) of the donor strain (i.e. the strain containing the marker to be moved) was added to 100 µl of phage suspension. $CaCl_2$ was added to a final concentration of 10 mM. The mixture was left at room temperature for 5 minutes, before adding to 7 ml of soft agar (SA), prewarmed to 42 °C. This mixture was then poured over an LA plate and incubated, with lids uppermost, overnight at 37 °C. The following day the phage were harvested by scraping off the soft agar into a sterile tube. This was centrifuged at 2880 x g for 10 minutes at room temperature (Eppendorf 5810R Centrifuge). The mixture was transferred to a sterile Bijou tube, 500 µl of chloroform was added and the resulting phage suspension stored at 4 °C.

2.6.5.2 Transduction into recipient cells

An overnight culture of the recipient cells (i.e. the cells into which the marker is being moved) was diluted and allowed to grow to mid log phase (OD_{600} ~ 0.2). The cells were centrifuged at 2880 x g for 10 minutes at room temperature (Eppendorf 5810R Centrifuge). The resultant pellet was resuspended in 1 ml of absorption fluid (10:1 ratio of distilled water and LB + 1 mM $CaCl_2$). The mixture was added to 150 µl of phage suspension and incubated at 37 °C for 30 minutes. 10 µl of 5% (w/v) sodium citrate was added and the resultant mixture was spread onto selective plates containing 0.05% w/v sodium citrate. The plates were incubated overnight at 37 °C. Colonies were purified of phage by streaking onto selective LA plates several times, and then screened for the required phenotype.

2.7 DNA manipulation

2.7.1 Plasmid purification

LB medium (10 ml) with appropriate antibiotic was inoculated with a single *E. coli* colony and incubated overnight with shaking at 120 rpm at 37 ºC, or 30 ºC for temperature sensitive strains. Plasmids were extracted according the manufacturer's instructions using the Qiaprep Spin Miniprep Kit, Qiagen™.

2.7.2 Polymerase chain reaction (PCR)

After P1 transduction had been performed to move *TnaA-GFP: Km*^{R} into BW25113 and BW25113 ∆ *rpoS*, PCR was performed to ensure BW25113 remained wild-type for RpoS, and that BW25113 ∆ *rpoS* retained the RpoS deletion. Primers were obtained from Sigma-Aldrich, and used amplify the RpoS gene .

Name of primer	**Sequence 5' -3'**
rpoS forward	TTCACGGGTGAGGCCAATTT
rpoS reverse	GACGCGACTCAGCTTTACCT

Table 2.7: Primers used to amplify the *rpoS* gene

Component	Concentration	Volume (x 8.5 master mix)/ µl
rpoS forward primer	10 mM	8.5
rpoS reverse primer	10 mM	8.5
dNTPs	10 mM	8.5
Thermopol buffer	X5	17
Sterile distilled water	-	85

Table 2.8: Master mix used for PCR

Master mix (15 µl) was aliquoted in PCR tubes. Candidate colonies were resuspended in 5 µl of sterile distilled water and added to master mix. Taq polymerase (1 µl) was added to each tube and the mixture subjected to cycling conditions shown below:

Initial denaturation: 94 °C for 2 minutes

Denaturation: 94 °C for 30 seconds
Annealing: 55 °C for 30 seconds
Extension: 72 °C for 30 seconds
Repeated 30 times

Final extension: 72 °C for 2 minutes
The resultant PCR product was analysed by gel electrophoresis.

2.7.3 Gel electrophoresis.

Electrophoresis through 1 % (w/v) agarose gels in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) was used to analyse PCR products. Samples were loaded after addition of 5 x loading dye (Sigma) and gels were run at 10 volts cm^{-1}. Gels

were stained using Ethidium Bromide (100 µg ml $^{-1}$), then visualized by UV transillumination.

2.8 Biochemical Assays

2.8.1 Kovacs assay for indole

Kovacs Solution:
10 g p-dimethylamino-benzaldehyde
150 ml iso-amyl alcohol
50 ml hydrochloric acid (analytical grade reagent)

HCl-amyl alcohol solution:
150 ml iso-amyl alcohol
50 ml hydrochloric acid (analytical grade reagent)
Both solutions were wrapped in aluminium foil to protect from light and stored at room temperature.

2.8.1.1 Measurement of external indole

To assay indole in a culture supernatant i.e. the external indole, a sample (1 ml) of culture was removed, the OD_{600} measured and the sample centrifuged to pellet cells at 11337 x g for 15 seconds (Eppendorf Minispin microfuge). The resulting supernatant was removed and assayed as such, 300 µl of Kovacs solution was added to the supernatant for 2 minutes. A 50 µl portion was removed and added to 1 ml of HCl-amyl alcohol solution. The absorbance at 540 nm was measured (Gene Quant 1300, GE Spectrophotometer). The concentration of indole in the supernatant was calculated using a calibration curve.

2.8.1.2 Measurement of cell associated indole

To assay indole contained within the cell pellet, i.e. cell associated indole, a modified method was used. A sample (1 ml) from a growing culture was removed, the OD_{600} measured and the sample centrifuged at 11337 x g for 15 seconds. The supernatant was discarded, 300 µl of Kovacs Reagent was added to the cell pellet for 2 minutes. This achieved cell lysis as well as the Kovacs reaction with the indole. This mixture was pipetted onto 1 ml LB before a 50 µl portion was removed and added to 1 ml of HCl-amyl alcohol solution. The absorbance at 540 nm was

measured (Gene Quant 1300, GE Spectrophotometer). The concentration of indole was calculated using a calibration curve.

Using the concentration of indole obtained from the calibration curve allowed calculation of the number of moles of indole present in the cell pellet. Using the OD_{600} obtained, we can calculate the total bacterial cell volume contained in the pellet (Volkmer and Heinemann, 2011) and hence the cell associated indole concentration.

2.8.2 Measurement of the affinity of cells for indole

LB medium (10 ml), was inoculated with a single colony of BW25113 Δ*tnaA* and incubated overnight with shaking at 120 rpm at 37 ºC. The OD_{600} was measured. Samples (1ml) of the culture were aliquoted and known concentrations of indole were added externally. The mixture was vortexed and immediately centrifuged for at 11337 x g for 15 seconds (Eppendorf Minispin microfuge). The supernatant was discarded and the cell pellet was assayed as such described in section 2.8.1.2.

2.8.3 Measurement of *TnaA–GFP* expression: fluorescence intensity

Appropriate medium (10 ml) was inoculated with a single *E. coli* colony and incubated overnight at 37 ºC with shaking at 120 rpm. Overnight cultures were diluted to an OD_{600} of 0.05 in appropriate medium and incubated at 37 ºC with shaking at 120 rpm.

Samples were removed, the OD_{600} measured and the GFP fluorescence in the samples was measured by using 480 nm excitation and 510 nm emission (Cary Eclipse Fluorescence spectrophotometer).

2.8.4 Indigo assay for indole

The strain to be assayed was transformed with the pStyABB plasmid. A single colony was inoculated in 10 ml of LB medium containing carbenicillin (100 µg ml^{-1}) and incubated overnight with shaking at 120 rpm at 37 ^{o}C. Samples (1 ml) were removed and the cells pelleted by centrifugation for at 11337 x g for 15 seconds (Eppendorf Minispin microfuge).The supernatant was discarded and the pellet was resuspended in 1 ml DMF (dimethyl formamide). This dissolved the indigo contained in the cells. The mixture was pelleted again by centrifugation for at 11337 x g for 15 seconds (Eppendorf Minispin microfuge) to remove cell debris. The

absorbance of the remaining supernatant was measured at 610 mm (Gene Quant 1300, GE Spectrophotometer).

Chapter 3

The role of indole production at the onset of stationary phase.

3.1 Introduction

A recent review by Lee and Lee (2010) identified over 85 species of bacteria which are able to make indole. These include both Gram-negative and Gram-positive species, including pathogenic strains. Despite this, indole production has historically been used as a diagnostic marker to identify *E. coli* specifically (Smith, 1897).

Indole is produced by tryptophanase (TnaA) which is encoded by the *tnaA* gene. Tryptophanase converts tryptophan into indole, pyruvate and ammonia (Newton and Snell, 1965). Tryptophanase expression is under a series of stringent controls in *E. coli,* including catabolite repression, transcriptional repression and attenuation (reviewed in the Introduction). The outcome of these controls is that tryptophanase, and therefore indole, can only be made in late exponential phase, when glucose levels are low and tryptophan levels are high.

E. coli makes very little indole during exponential growth (Chant and Summers, 2007). However *tnaA* transcription is strongly up-regulated by the stationary phase sigma factor RpoS (Lacour and Landini, 2004), and consequently indole production rises as cells approach stationary phase (Chant and Summers, 2007). The final concentration of indole in stationary phase culture depends upon the amount of tryptophan in the growth medium. In LB medium, free tryptophan can range between 0.5-1 mM (Li and Young, 2013, Sezonov *et al*., 2007) and therefore supernatant concentrations of indole also typically reach 0.5-1 mM (Li and Young, 2013).

Persistent exposure to 0.5-1 mM of indole has been shown to have a wide range of effects on *E. coli*, including regulation of biofilm formation, acid resistance and virulence (reviewed in the Introduction). Indole clearly has a variety of important and possibly beneficial roles in the cell. However, there is some suggestion in the literature than indole production can put cells at a disadvantage. The phenotype of mutant cells which have had tryptophanase knocked out and therefore cannot produce indole (Δ *tnaA*) has been studied. Zhang and Hong (2009) showed that over an 8 hour period, Δ *tnaA* cells grew to a higher density than wild-type, indole producing counterparts. Furthermore they reported that they were able to restore wild-type behaviour by external addition of 400 µM indole to the culture supernatant Δ *tnaA* cells. They suggested that not making indole led to a postponement of the

entry into stationary phase of the mutant cells, proposing that indole is an important stationary phase signalling molecule.

This report of indole non-producing cells growing to a higher cell density seems somewhat paradoxical; it suggests that not making indole allows cells to grow to a higher density, conferring an advantage to the mutant cells over their wild-type counterparts. Furthermore the authors suggest that indole production results in early entry to stationary phase, but do not suggest a mechanism by which low levels of indole are able to achieve this. The concentrations used (400 µM) are not sufficient to affect cell division *via* the ionophore mechanism (Chimerel *et al*., 2012) or inhibit growth (Chant and Summers, 2007).

The work detailed in this chapter aimed to revisit the functional significance of indole production on entry to stationary phase. The kinetics of indole production were studied in detail and the role of indole production was assessed, not only on stationary phase entry but also during subsequent periods of starvation.

3.2 External indole accumulates rapidly on entry into stationary phase

An overnight culture of BW25113 was diluted into fresh LB medium in triplicate to an OD_{600} of 0.05. These cultures were grown at 37 °C with shaking for 300 minutes. Initially, the OD_{600} and concentration of indole in the supernatant were measured once every 60 minutes. After 120 minutes, on the approach to stationary phase, samples were removed every 5 minutes, and the OD_{600} and concentration of indole in the supernatant were measured (Fig. 3.1A)

The cells grew exponentially for approximately 120 minutes. After this time, growth started to slow significantly, with residual growth observed for the remaining 180 minutes. Therefore, the period from 120 minutes onwards corresponds to the transition into stationary phase.

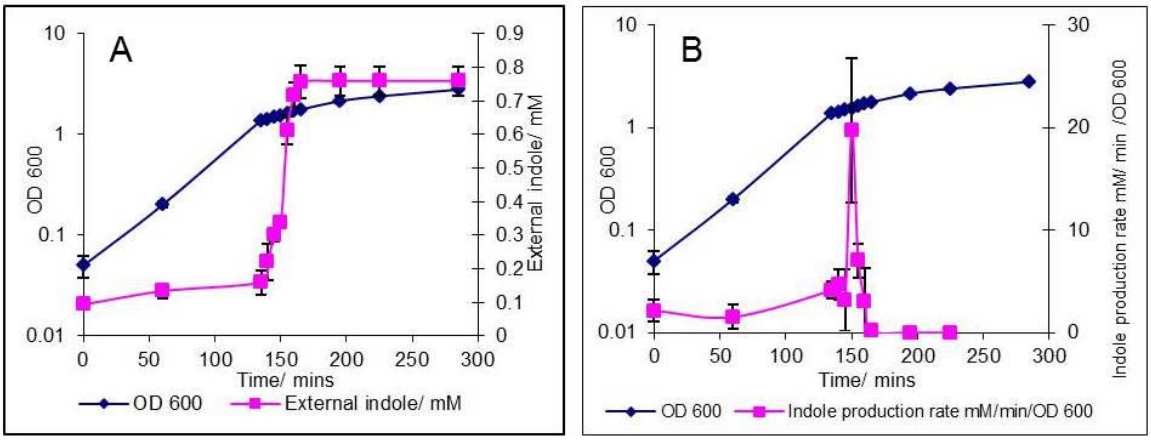


Figure 3. 1 Indole accumulates rapidly in the supernatant during the onset of stationary phase, due to an increased production rate. A culture of BW25113 cells was sampled, the OD $_{600}$ was measured and the supernatant was assayed for external indole using Kovacs assay (A). Data shown are the mean values ± standard deviation for three independent repeats. These data were used to calculate indole production rate (B).

The concentration of indole in the supernatant was low in late exponential phase (<0.2 mM). The concentration rose rapidly to approximately 0.75 mM, when the culture reached an OD $_{600}$ of 1.0-1.5. This corresponded to the period of transition between exponential growth and stationary phase.

Over this 30 minute period, there was an approximately 5-fold increase in indole in the supernatant. This implies an increased production rate *per* cell, rather than simply an increase in the number of cells producing indole at a constant rate, as the OD $_{600}$ of the culture increased only 1.4-fold during this period. Consistent with reports elsewhere (Li and Young, 2013), the supernatant concentration reached a maximum of 0.75 mM once in stationary phase.

From these data, it was possible to calculate the indole production rate per cell. The change in indole concentration between two consecutive samples was divided by the change in OD $_{600}$ and the time elapsed. This variation in the indole production rate (mM/OD $_{600}$/ minute) over the course of the experiment is shown in Fig. 3.1B.

The indole production rate remained low for most of the experiment, however it peaked sharply at the transition between exponential and stationary phase when it increased approximately 5-fold over a 15 minute period, before quickly reducing again.

3.3 Non indole producing mutants grow to a higher cell density than indole producers during stationary phase entry

To assess the role of rapid synthesis of indole at the onset of stationary phase, cell density (OD_{600}) and cell numbers (CFU X 10^6/ ml) of BW25113 and BW25113 Δ*tnaA* (a tryptophanase knock-out that cannot produce indole) cultures were compared over 24 hours.

Overnight cultures of BW25113 and BW25113 Δ*tnaA* were diluted into fresh LB medium in triplicate to an OD_{600} of 0.05. These cultures were grown at 37 °C with shaking for 24 hours. The cell density (by measuring OD_{600}) and cell numbers (by diluting appropriately, plating onto LA plates and counting colonies the following day to obtain CFU X 10^6/ ml) were measured. The viability (CFU X 10^6/ ml/ OD_{600}) of the cultures was also calculated (Table 3.1). Both the density and numbers of the BW25113 Δ *tnaA* cells were significantly higher than the BW25113 cells after 24 hours. The viability was the same for the two strains.

This is consistent with the finding of Zhang and Hong (2009) who showed that after 8 hours, when the cells had entered stationary phase, the density of a BW25113 Δ *tnaA* culture is significantly higher than the density of a BW25113 culture.

Table 3.1 Culture density (OD_{600}), colony forming units (CFU x 10^6/ ml) and viability (CFU X 10^6/ ml/ OD_{600}) of BW25113 and BW25113 Δ *tnaA* cultures after 24 hours.

Strain	OD_{600} [A]	CFU x 10^6/ ml [A]	Viability (CFU X 10^6/ ml/ OD_{600})
BW25113	3.22±0.11	2.31±0.01	0.72±0.12
BW25113 Δ *tnaA*	4.36±0.09	3.37±0.40	0.77±0.49

[A]: . Data shown are the mean values ± standard deviation for three independent repeats

3.4 Addition of 1 mM external indole to the non indole producing mutant is insufficient to restore the wild-type phenotype

Zhang and Hong (2009) also monitored the density of a culture of BW25113 Δ *tnaA* cells with 400 µM indole added to the supernatant. They found that addition of 400 µM indole (at an undefined point of growth) to the mutant culture caused it to enter stationary phase at the same time as the wild-type culture, and the same density to be reached after 8 hours, both of which were significantly lower than the BW25113 Δ *tnaA* cells with no indole added. They therefore concluded that external indole is key to stationary phase entry, as when it is restored to wild-type levels in the mutant cultures, the wild-type phenotype is restored.

To better mimic the change in indole concentration observed in wild-type cultures, 1 mM of external indole was added, when the cells were entering stationary phase at an OD_{600} of 1.5. This was different from the method used by Zhang and Hong (2009) where 400 µM of indole was added at an undefined point of growth.

Overnight cultures of BW25113 and BW25113 Δ*tnaA* were diluted into fresh LB medium in triplicate to an OD_{600} of 0.05. Three BW25113 and six BW25113 Δ*tnaA* cultures were grown at 37 °C with shaking. When the OD_{600} of the BW25113 Δ*tnaA* cultures reached approximately 1.5, either 1 mM indole or the corresponding volume of ethanol (the solvent indole was dissolved in) was added to the culture. After 24 hours the cell density (by measuring OD_{600}) and cell numbers (by diluting appropriately, plating onto LA plates and counting colonies the following day to obtain CFU X 10^6/ ml) were measured. The viability (CFU X 10^6/ ml/ OD_{600}) of the cultures was also calculated (Table 3.2).

Both the density and cell numbers of the BW25113 Δ *tnaA* + 1 mM indole culture were indistinguishable from the BW25113 Δ *tnaA* culture. Cell density and cell numbers in the BW25113 Δ *tnaA* and BW25113 Δ *tnaA* + 1 mM indole cultures were significantly higher than cell density and cell numbers in the BW25113 culture. There were no significant differences in viability of the three culture conditions.

The cell density data are not consistent with the Zhang and Hong (2009) finding that external indole could restore the wild-type phenotype to a mutant culture. This may reflect the differences in the way the external indole was provided, but this result was highly reproducible, the mutant phenotype was never restored by adding exogenous indole.

Table 3.2 Culture density (OD_{600}), colony forming units (CFU x 10^6/ ml) and viability (CFU X 10^6/ ml/ OD_{600}) of BW25113, BW25113 Δ *tnaA* BW25113 Δ*tnaA* + 1 mM Indole cultures after 24 hours.

Strain	OD_{600} [A]	CFU x 10^6/ ml [A]	Viability (CFU X 10^6/ ml/ OD_{600})
BW25113	3.22±0.11	2.31±0.01	0.72±0.12
BW25113 Δ *tnaA*	4.36±0.09	3.37±0.40	0.77±0.49
BW25113 Δ*tnaA* + 1 mM Indole	4.12±0.10	3.50±0.45	0.85±0.55

[A]: . Data shown are the mean values ± standard deviation for three independent repeats

3.5 Indole production is important for long term viability

The production of indole at the onset of stationary phase appeared to confer no advantage to BW25113 cells over a 24 hour period. Indeed it seemed that indole production confers a growth disadvantage to wild-type cells. In order to see if this apparent disadvantage for indole producers persisted, the effect of indole production on cell density and numbers was examined over a more extended period.

Overnight cultures of BW25113 and BW25113 Δ*tnaA* were diluted into fresh LB medium in triplicate to an OD_{600} of 0.05. Three BW25113 and six BW25113 Δ*tnaA* cultures were grown at 37 °C with shaking. When the OD_{600} of the BW25113 Δ*tnaA* cultures reached approximately 1.5, either 1 mM indole or the corresponding volume of ethanol was added to the culture. After 24 hours the cell density (by measuring OD_{600}) and cell numbers (by diluting appropriately, plating onto LA plates and counting colonies the following day to obtain CFU X 10^6/ ml) were measured. The cultures were kept shaking at 37°C for a further 9 days. Samples were taken every 2-3 days and the cell density (by measuring OD_{600}) and cell numbers (by diluting appropriately, plating onto LA plates and counting colonies the following day to obtain CFU X 10^6/ ml) were measured (Fig. 3.2)

For the first 3 days the density of BW25113 cultures was significantly lower than the BW25113 Δ*tnaA* cultures, confirming the previous observation (Fig. 3.2A). However over the subsequent 7 days the OD $_{600}$ of the mutant culture declined, while the density of the wild-type continued slowly to increase. Consequently, after 10 days in stationary phase, it was the wild-type culture that had the higher density.

The cell number data showed that for the first 3 days BW25113 cultures were significantly lower than the BW25113 Δ*tnaA* cultures (Fig. 3.2B). However over the subsequent 7 days the cell numbers in the mutant culture declined rapidly, whilst the cell numbers of the wild-type declined less quickly. Consequently, after 10 days in stationary phase, it was the wild-type culture that had the higher cell numbers.

The BW25113 Δ*tnaA* + 1 mM indole cultures were indistinguishable from the BW25113 Δ*tnaA* cultures at all times. Initially the BW25113 Δ*tnaA* + 1 mM indole cultures had a higher density and cell number than the wild-type culture, then at a lower density and cell number than the wild-type culture.

During 10 days in stationary phase, the number of cells in the mutant cultures (with and without 1 mM indole added) decreased by approximately 75%. However the wild-type culture cell numbers decreased by only 30%. This suggests that indole production may be important in maintain cell numbers in long term stationary phase.

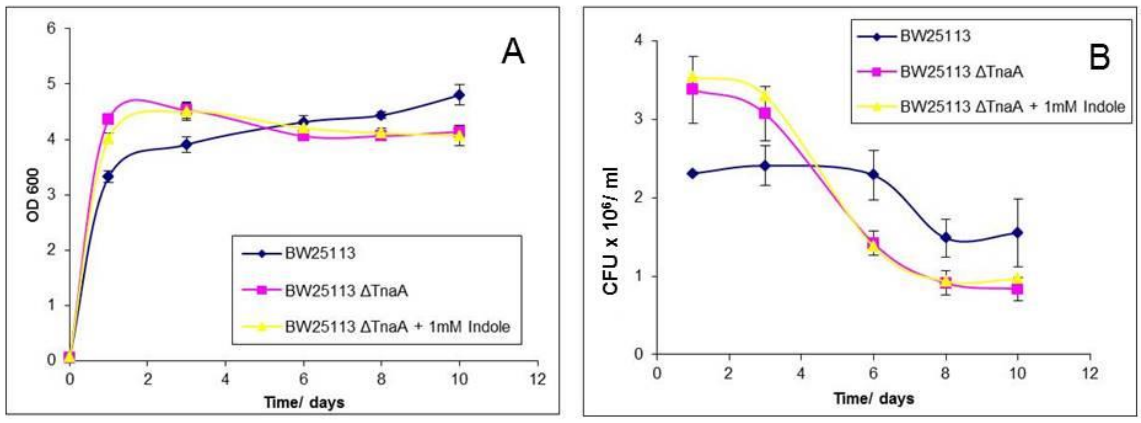


Figure 3.2 Non indole producing mutants initially grow to a higher density and are more viable than their wild type counterparts, but are significantly less viable in the long term . The density (OD $_{600}$: A) and cell numbers (CFU x 10^6/ ml :B) of BW25113, BW25113 Δ *tnaA* and BW25113 Δ *tnaA* cells with 1 mM indole added were assessed over 10 days. Data shown are the mean values ± standard deviation for three independent repeats.

3.6 Discussion

The results presented here show that in the short term (up to 3 days) the cell density and numbers in a wild-type culture are lower than the mutant culture. This is consistent with previously published findings of Zhang and Hong (2009). This result seemed to show that production of indole conferred a significant disadvantage to cells, which seemed somewhat paradoxical.

This experiment was extended to assess cell density and cell numbers over 10 days. This showed that, in the longer term, indole producing cells had an advantage as cell density and cell numbers in wild-type cultures are significantly better maintained than in mutant cultures, with or without 1 mM indole added. This extension of the experiment has enabled this apparent indole production paradox to be solved. Indole production is important as in the long term numbers of wild-type cells are effectively maintained.

The failure of 1 mM indole to restore the long-term maintenance of the Δ *tnaA* mutant was initially surprising and contradicted previously published work. The experiment described in section 3.4 was designed to closely reproduce the external indole concentrations experienced by wild-type cells to the mutant culture. 1 mM indole was added when the mutant cells reached an $OD_{600} \approx 1.5$, the density at which indole appears rapidly in a wild-type culture.

Despite, this, the failure to complement was highly reproducible. This suggests that indole *production,* not simply the presence of indole in the supernatant, is key to ensuring long term maintenance of the culture in extended stationary phase. Indole production by wild-type cells during stationary phase entry slows their growth. However, low indole concentrations (1-2 mM) have no effect on growth (Chant and Summers, 2007). A higher concentration (3-5 mM) would be required to have this effect. Because indole production during stationary phase entry is very rapid, it is possible that a high concentration of indole exists inside the cells during the increased production period. If a sufficiently high concentration were reached, this might explain the slowing of growth by the wild-type strain. This possibility will be explored in the next chapter.

Chapter 4

Increased indole production rates results in high levels of cell associated indole

4.1 Introduction

Chapter 3 showed that indole production is rapidly upregulated in late exponential phase and that this influences the entry of cells into stationary phase. Initially a wild-type culture has a lower optical density and cell numbers than an indole non-producing mutant. However over 10 days in stationary phase the wild-type cells show a much higher survival rate than the mutants.

Exogenous addition of 1 mM indole to the culture supernatant of mutant cells was insufficient to restore the wild-type behaviour. Whilst this was initially surprising, it has been shown previously that low (1-2 mM) concentrations of indole had no effect on cell growth, so perhaps 1 mM indole would not be expected to restore the wild-type kinetics of growth on entry into stationary phase. Higher levels of exogenous indole (3-5 mM) need to be added to the culture supernatant to affect growth and cell division (Chant and Summers, 2007, Chimerel *et al.*, 2012).

One way to achieve higher levels of indole inside cells could be through an increased production rate. An increased indole production rate at the onset of stationary phase has been demonstrated previously (Fig. 3.1). With this in mind a hypothesis is proposed, this is referred to as the ‘pulse’ signalling hypothesis (Fig. 4.1). During exponential growth cells make very little indole. On entry into stationary phase, indole production rate increases sharply. It seems possible that a sufficiently increased indole production rate could result in a brief time where the internal level of indole is substantially higher than that outside. It is plausible that if indole is being made faster than it can diffuse out of the cell; a period could exist with higher internal levels of indole. When the production rate subsequently falls the indole will diffuse out of the cells into the surrounding supernatant. A further consideration here is that volume of cells is very small compared to the overall culture volume. This could contribute to high local concentrations of indole in the cells before it diffuses away and dilutes rapidly into the culture supernatant.

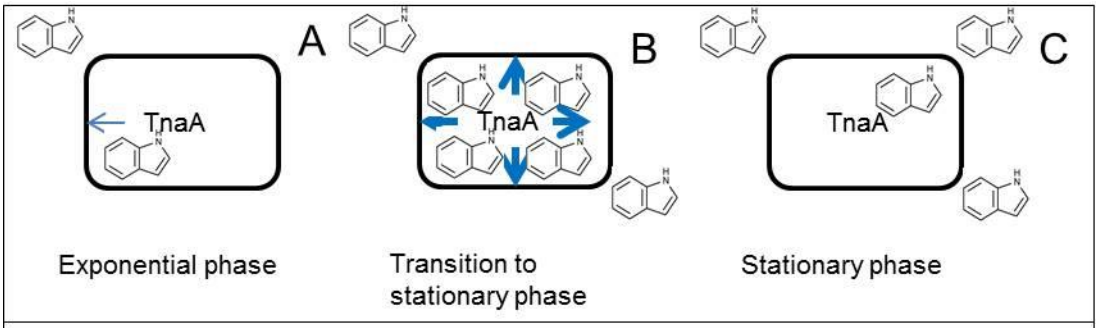


Figure 4.1 The 'pulse' signalling hypothesis. During exponential phase very little indole is made by cells (A). At the onset of stationary phase, indole production rates increase. This results in a period where internal levels of indole are substantially higher than those outside (B). Eventually all indole will diffuse out of the cell, resulting in indole present in the supernatant during stationary phase (C).

If the level of indole experienced by cells during the period of rapid production was sufficient to affect cell growth and division (3-5 mM) this may explain why cells which produce indole exhibit the wild-type stationary phase entry phenotype. This would explain why addition of 1 mM indole to culture supernatants of mutant cells is insufficient to restore the wild-type phenotype.

This chapter will focus on quantifying internal amounts of indole, and will ask whether internal levels are sufficient to affect growth on entry into stationary phase.

4.2 Cell associated indole is elevated rapidly before entry into stationary phase

The hypothesis predicts that when the indole production rate is highest, there will be a brief period with high internal levels of indole. This level of indole would subsequently fall, as the indole diffuses out of the cells and into the surrounding supernatant. Directly measuring intracellular indole during the transition from exponential to stationary phase would indicate if this hypothesis was valid.

The Kovacs assay was modified to allow the measurement of cell associated concentrations of indole. Cell associated indole represents an average value of the indole contained in both the cytoplasm and cell membrane.

The following changes were made to Kovacs assay to allow measurement of the concentration of cell associated indole instead of the conventional measurement of indole in the supernatant as before. A 1 ml sample of culture was centrifuged to pellet the cells. The supernatant was removed and discarded. Addition of Kovacs reagent directly to the cell pellet allowed cell lysis and reaction with the indole present. The concentration of indole was calculated using a standard curve.

Using the concentration of indole obtained from the calibration curve allowed us to determine the number of moles of indole present in the cell pellet *(Ip).* Using the OD_{600} of the culture, we can calculate the total bacterial cell volume *(Vc)* contained in the pellet (Volkmer and Heinemann, 2011) and hence the cell associated indole concentration *(C_A)* as shown in equation 1.

$$C_A = \frac{I_p}{V_c}$$ Equation (1)

An overnight culture of BW25113 was diluted into fresh LB medium, in triplicate, to an OD_{600} of 0.05. These cultures were grown at 37 °C with shaking for 300 minutes. Initially, the OD_{600} and concentration of indole associated with the cells were measured once every 60 minutes. After 120 minutes, on the approach to stationary phase, 1 ml samples were removed every 5 minutes, and the OD_{600} and the concentration of cell associated indole were measured (Fig. 4.2).

The cells grew exponentially for approximately 120 minutes. After this time the growth started to slow significantly, with slow residual growth observed for the remaining 180 minutes. Therefore, the period from 120 minutes onwards corresponds to the transition into stationary phase.

During exponential phase the cell-associated indole remained constant approximately 15 mM. At an OD_{600} of approximately 1.5, the cell-associated indole then began to increase quickly. This increase in cell associated indole was observed for approximately 30 minutes, reaching a peak of approximately 60 mM. This corresponds to the entry of stationary phase. After this brief period the cell associated indole decreased quickly at first, then more gradually, back to approximately 15 mM.

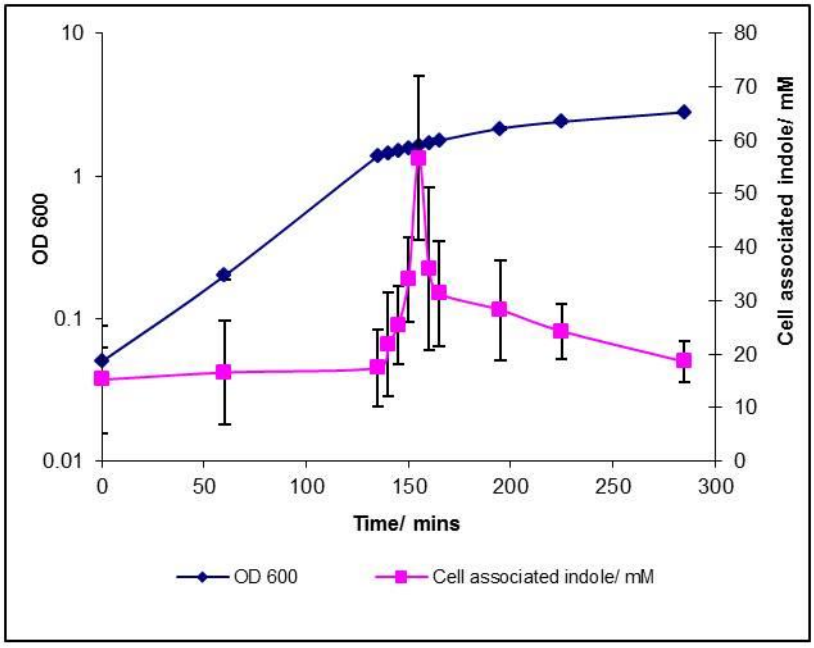


Figure 4.2 Cell associated indole accumulates rapidly at the onset of stationary phase. A culture of growing BW25113 cells was sampled. The OD_{600} was measured and the samples were centrifuged to pellet cells. The resultant cell pellet was assayed for indole using Kovacs assay. Data shown are the mean values ± standard deviation for three independent repeats.

During exponential phase, when the supernatant concentration is approximately 0-0.2 mM (Fig. 3.1), the calculated cell associated indole concentration is between 5-20 mM approximately. This observation, that the cell associated indole concentration was always significantly higher than the external amount of indole was initially surprising. However, this can be explained as the cell associated indole concentration represents an average value of indole in both the cytoplasm and the cell membrane.

It seemed plausible that if indole had a much higher affinity for the cell membrane, this would help explain the high values obtained. It has previously been predicted that indole should have a high affinity for a hydrophobic solvent (Kamaraju *et al.,* 2011). The partition coefficient (P) describes the distribution of a molecule in two immiscible phases, e.g. octanol and water from a chemical point of view, or, for a biologist, the cell membrane and cytoplasm. A theoretical value of log (P) = 2.17 for octanol-water partitioning for indole had been calculated by Kamaraju *et al.* (2011). This corresponds to indole having an approximate 100 fold higher affinity for octanol compared to water. This is due to the hydrophobic nature of the aromatic ring of

indole. It therefore seemed likely that the high values of cell associated indole might reflect the relatively large amount of indole in the cell membrane.

Our collaborator from the Cavendish Laboratory, Silvia Hernández-Ainsa, performed a series of measurements to measure the partition coefficient of indole between water and *E. coli* total lipid extract. A value of log (P) = 1.95±0.12 (n=9) was obtained, which is similar to the predicted value from Kamaraju *et al.,* (2011). This means that indole has an approximately 90 fold higher affinity for the cell membrane, compared to the aqueous cytoplasm or supernatant. Therefore, the high values obtained by the cell associated assay, will reflect the high level of indole present in the cell membrane.

4.3 Cell associated indole reaches a level which is likely to be sufficient to inhibit cell division

The fact that *E. coli* cells, by virtue of their lipid membranes, always have a greater affinity for indole than their aqueous surroundings complicates understanding of the data in Fig. 4.2. Since the previous experimental investigations of the effects of indole on the growth and division of *E. coli* have been conducted by adding indole to the supernatant (Chant and Summers, 2007; Chimerel *et al*., 2012), it is helpful to know how much indole would have to be added to the supernatant to achieve the observed cell associated concentrations.

This was investigated by addition of a range of known concentrations of indole to the supernatant of indole non-producing cells. The cells, by virtue of the lipid membranes, will act to ‘soak up’ the indole. The resulting cell associated indole can then be measured. A relationship between the supernatant indole added and the measured cell associated indole is useful as it allows interpretation of cell associated indole concentrations in terms of supernatant equivalent concentrations, which in turn allows sensible conclusions about the likely effects on growth and cell division to be made.

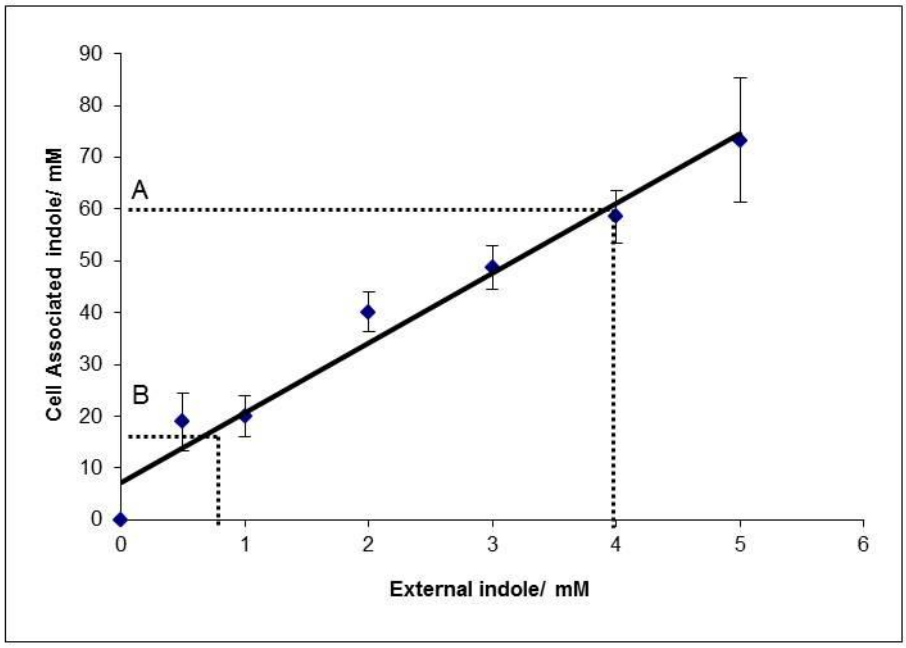


Figure 4.3 The relationship between cell associated concentrations of indole and supernatant concentrations of indole. Known concentrations of indole were added to the supernatant of stationary phase BW25113 Δ *tnaA* cells, the mixture was vortexed and spun down, and the resultant pellet assayed for indole using Kovacs assay. Dotted line (A) indicates the cell associated indole at the peak of the pulse and the corresponding supernatant concentration and (B) indicates the cell associated indole when cells are in stationary phase and the corresponding supernatant concentration. Data shown are the mean values ± standard deviation for at least three independent repeats.

A single colony of BW25113 Δ*tnaA (*an indole non-producing mutant) was inoculated in LB medium and grown overnight at 37 °C with shaking. Samples of the overnight BW25113 Δ*tnaA* culture (1 ml) were removed, the OD_{600} measured and known concentrations (0-5 mM) of indole were added to the supernatant. The mixture was vortexed, cells were harvested by centrifugation and the pellet assayed for cell-associated indole as described previously. This allowed a graph of cell associated indole against external indole added to be plotted (Fig. 4.3).

There is a linear relationship between the concentration of indole in the supernatant and the concentration of cell associated indole. These data can be used in two ways. Firstly, it can be deduced from Fig. 4.3 that a supernatant concentration of 0.8 mM (typical of stationary phase cultures of wild-type cells) gave a cell-associated concentration of approximately 15 mM. This level of cell associated indole is indeed what is observed for stationary phase cells (Fig. 4.2).

Secondly, a cell associated concentration of 60 mM (the maximum detected during stationary phase entry, Fig. 4.2) resulted from a supernatant concentration of

approximately 4 mM. This is significant as when concentrations between 3-5 mM are added to the supernatant of cells this is sufficient to inhibit growth and cell division (Chant and Summers, 2007; Chimerel *et al*., 2012).

To confirm that the high concentrations of cell-associated indole are not an artefact of the Kovacs assay, the relationship between cell-associated and supernatant indole was investigated by an independent technique by our collaborator at the Cavendish Laboratory, Jehangir Cama. The technique used the intrinsic fluorescence of indole to measure its concentration. However, the fluorescence of indole can only be measured reliably when it is in solution, not when it is associated with cells, as many other biological molecules also shown intrinsic fluorescence at similar wavelengths. Therefore, the approach taken was to measure the removal of indole from the supernatant by cells.

Known concentrations (0-1 mM) of indole were added to the supernatant of stationary phase BW25113 Δ *tnaA* cells (1 ml), the mixture was vortexed and the cells harvested by centrifugation, as above. The amount of indole removed from the supernatant by the cells was measured using an UV absorbance assay for indole.

When the external indole concentration added was 1 mM, a cell-associated concentration of 7±3 mM was obtained (8 repeats). This compares with a value of 17±3 mM from the Kovacs assay (Fig. 4. 3) The values obtained by the two assays although different, are within the same order of magnitude, suggesting that the high levels of cell associated indole is not an artefact of a specific technique.

It was initially thought that the difference between the cell associated indole concentrations between the two techniques may be a systematic error due to procedural differences between the assays. In the Kovacs assay the total amount of indole associated with the pellet is measured, and this includes extracellular indole in the spaces between cells in the pellet. The amount of indole in the pellet may therefore be overestimated. Since the UV absorbance assay measures cell associated indole indirectly it does not suffer from this source of error. The calculation outlined below allows us to correct for this error.

$$V_p = V_c + V_s = V_c + \frac{1}{2}V_c = \frac{3}{2}V_c \qquad \text{Equation (2)}$$

Equation 2 states that that the volume of the pellet is equal to the sum of the volume of the trapped supernatant and the volume of the cells. Reid and Frank (1966) stated that in a cell pellet, approximately 1/3 of the pellet will correspond to trapped supernatant.

$$C_p = \frac{I_p}{V_p} = \frac{2}{3} \times \frac{I_p}{V_c} = \frac{2}{3} C_A \qquad \text{Equation (3)}$$

$$\mathrm{C_p} = \mathrm{C}_c \times \mathrm{R}_{Vc} + \mathrm{C}_s \times \mathrm{R}_{Vs} = \frac{2}{3} C_c + \frac{1}{3} C_s \qquad \text{Equation (4)}$$

where C_A is the apparent cell associated indole concentration in the pellet, I_p is the number of moles of indole in the pellet, V_c is the volume of the cells, V_p is the volume of the pellet and V_s is the volume of the supernatant, C_p is the concentration of indole in the pellet, C_c is the concentration of indole in the cells and C_s is the concentration of indole in the trapped supernatant, R_{Vp} is the volume fraction of the cells in the pellet ($R_{Vp}=\frac{2}{3}$)and R_{Vs} is the volume fraction of the supernatant in the pellet ($R_{Vs}=\frac{1}{3}$), taken from Reid and Frank (1966).

In Fig. 4.3, 4 mM indole in the supernatant resulted in 60 mM cell associated indole. When the correction is applied, the actual cell associated indole concentration is shown to be 58 mM rather than 60 mM. Thus, the contribution of indole from trapped supernatant to the cell associated indole concentrations is negligible and well within the experimental errors of the Kovacs assay.

4.3 Discussion

The data presented in this chapter show that cell associated indole peaks sharply at the onset to stationary phase. This happens immediately after an increased indole production rate being observed (Fig. 3.2). It therefore seems likely that the indole pulse is caused directly by the increased indole production rate.

Cell associated indole reached approximately 60 mM, a value which reflects the average amount of indole in both the cell membrane and cytoplasm. This increase in cell associated indole was observed for approximately 30 minutes. It is important to note that this pulse duration is an average for all cells in the culture. If there is significant population heterogeneity in the time of pulsing, the pulse duration in

individual cells may be shorter and the maximum concentration higher than the values presented here.

To aid interpretation of the likely effect of 60 mM cell associated indole, cell-associated concentrations corresponding to supernatant indole in the 0-5 mM range were measured. This showed that when the cell-associated concentration is 15 mM (as in late stationary phase, Fig. 4.2), the supernatant-equivalent concentration is approx. 0.8 mM, which is very similar to the actual supernatant concentration observed in a stationary phase culture of wild-type cells (Fig. 3.1). However, when the cell-associated concentration is 60 mM at the peak of the pulse (Fig. 4.2), the supernatant-equivalent concentration is 4 mM (Fig. 4. 3). This is significant as concentrations of indole added to the supernatant in this range (between 3-5 mM) are sufficient to inhibit growth and cell division (Chant and Summers, 2007; Chimerel *et al*., 2012).

Whilst the values obtained for using two independent techniques were within the same order of magnitude there was a clear difference between the values obtained. It was initially thought that this could be due to the presence of trapped supernatant in the cell pellet in the Kovacs assay, but this effect was shown to be negligible. However, if more data points corresponding to a range (2-5 mM) of external indole concentrations were obtained for the UV absorbance assay, it might be expected that all cell associated indole values would be lower than the values obtained using Kovacs assay, as for the 1 mM results reported above. If this was indeed the case, then the slope of the graph in Fig. 4.3 would be less steep. This would mean that a given value of cell associated indole would correspond to a higher value for external indole for the UV absorbance assay, than the Kovacs assay. For example, the cell associated value observed at the peak of the pulse, 60 mM, corresponds to approximately 4 mM external indole according to the Kovacs assay results. If the slope of the graph derived using the UV absorbance assay was indeed less steep, 60 mM cell associated indole would correspond to a higher external indole value. Therefore, the discrepancy between the two values is unlikely to affect the conclusion that, at the peak of the pulse, cell associated indole concentration reaches a level which is sufficient to affect cell division and growth.

It is clear from our data that the indole pulse is a non-equilibrium phenomenon. At the peak of the pulse, the concentration of cell-associated indole (60 mM; Fig. 4.2) is 150-fold higher than the supernatant concentration (0.4 mM; Fig. 3.1). As the

system returns towards equilibrium in stationary phase, the cell-associated concentration (15 mM) exceeds the supernatant concentration (0.8 mM) by less than 20-fold.

Measurement of the partition coefficient of indole between water and *E.coli* total lipid extract demonstrated that indole has an approximate 90 fold greater affinity for the cell membrane. Within the cell, the indole concentration in the membrane will be substantially higher than either the cytoplasmic or the overall cell-associated concentration.

In order to better understand quantitatively the distribution of indole in cells, a series of calculations were performed by Silvia Hernández-Ainsa:

> It was estimated that the lipid membrane accounts for 1/40th of the total cell volume, using the following calculations:
>
> The volume of the lipid membrane in *E.coli* (0.04 μm^3) was determined using equation (5) and applying simple geometrical calculations. In these calculations, *E. coli* is assumed to have a cylindrical shape. *l* is the length of *E.coli* (1.6 μm) (Volkmer and Heinemann, 2011), *r* is the estimated radius of the cylinder (0.55 μm) and *d* is the thickness of the lipid bilayer (around 5 nm, taken from Alberts, 2005).
>
> $$\text{Volume lipid membrane} = \left[\left(2\pi lr\right) + \left(2\pi r^2\right)\right] \times d \qquad \text{Equation (5)}$$
>
> The volume of the entire *E. coli* is 1.5 μm^3, so the lipid membrane represents 1/40th of the total cell volume.
>
> Therefore, as indole has approximately a 90 fold higher affinity for the cell membrane, but the cell membrane represents only 1/40th of the total cell volume, there will be approximately twice as much indole in the membranes as in the cytoplasm.
>
> The molecular ratio indole:lipid and the weight percentage indole:lipid were also estimated. The molecular ratio indole:lipid was estimated using Equation (7) (simplified from Equation (6)) where *d* is the density of the lipids (1000 g L^{-1}), V_{lipid} is the volume of the lipid membrane in a single *E. coli* (0.04 μm^3), M_{lipid} is the molecular weight of *E. coli* total extract lipids (811.5 g mol^{-1}), N_A is the Avogadro constant (6.023x10^{23}), C_{indole} is the concentration of

indole added to the supernatant (0.005 mol L^{-1}) and P is the *E. coli* lipids-buffer partition coefficient (92.9±24.5).

$$\frac{\text{Number indole molecules}}{\text{Number lipid molecules}} = \frac{P \times C_{indole} \times V_{lipid} \times N_A}{d \times V_{lipid} \times \left(\frac{1}{M_{lipid}}\right) N_A} \qquad \text{Equation (6)}$$

$$\frac{\text{Number indole molecules}}{\text{Number lipid molecules}} = \frac{P \times C_{indole} \times M_{lipid}}{d} \qquad \text{Equation (7)}$$

This gave a value of (0.4±0.1) for the molecular ratio indole:lipid.

However, the molecular weight of *E. coli* lipids is considerably greater than the molecular weight of indole, so it is useful also to express the ratio of indole:lipid by mass. The percentage in weight indole:lipid has been calculated multiplying Equation (7) by the ratio of molecular weights of indole:lipid, namely (117.2:811.5) to give a value 0.05:1

These results show that during the pulse a huge amount of indole exist briefly inside the cell membrane. It is suggested that the primary effect of this is to inhibit growth and cell division, *via* an ionophore base mechanism, on entry into stationary phase. However, it is possible that the indole pulse will affect the structure of the membrane and its properties in other ways. Furthermore the structure and function of membrane associated proteins is also likely to be affected by the indole pulse.

These results explain why, as shown in Chapter 3, addition of 1 mM external indole to mutant culture is unable to restore the wild-type phenotype of extended viability in stationary phase. Addition of 1 mM indole to mutant cells resulted in approximately 15 mM of cell associated indole, which is four fold lower than the pulse generated by indole production in wild-type cells. Cells must be producing indole themselves in order to experience the 60 mM cell associated indole ‘pulse’ at entry to stationary phase.

Using these results the pulse signalling hypothesis was further developed. At entry to stationary phase, indole production is rapidly upregulated, resulting in a 60 mM ‘pulse’ of indole associated with cells. The 60 mM cell associated indole is equivalent to 4 mM indole in the supernatant which is sufficient to slow growth and cell division. This hypothesis helps explain the different phenotypes of wild-type and indole non-producing mutants. It also suggests that the ‘pulse’ of indole initially

slows growth, potentially explaining why after 24 hours the wild-type cells have a lower density and cell numbers than mutants (Fig. 3.2). However, the ‘pulse’ of indole seems to provide some benefit to wild-type cells in the long term. It may help explain why wild-type cells are able to maintain cell density and numbers significantly better than mutant cells across an extended period (Fig. 3.2). A potential mechanism behind this could be that the pulse slows growth resulting in resources being conserved, which the cells are able to utilise in the long term.

In summary, the model suggests that it is the ‘pulse’ of cell associated indole, not low, external levels of indole which are key to determining stationary phase entry. The model also suggests that some long term benefit to the cells is conferred by the ‘pulse’ of indole. The subsequent chapters will focus on testing the assertions within the hypothesis and understanding the mechanism behind the ‘pulse’.

Chapter 5

Testing the pulse signalling hypothesis

5.1 Introduction

Chapters 3 and 4 have shown that *E. coli* indole production is upregulated on entry into stationary phase. This results in a brief and intense pulse of indole associated with the cells. The key to pulse signalling is the cell associated indole concentrations; low external levels of indole in the supernatant are irrelevant here. This cell associated indole pulse of 60 mM is equivalent to the effect of adding 4 mM indole to the supernatant of cells, which is sufficient to strongly inhibit growth and cell division (Chant and Summers, 2007, Chimerel *et al.,* 2012). This indole pulse, that slows growth and advances entry into stationary phase, is required for long term maintenance of the culture. It is suggested that by slowing growth, the indole pulse ensures that some resources are conserved on entry into stationary phase, which can ensure long term maintenance of the culture.

The two key assertions in this hypothesis are that

1. The pulse of indole regulates the timing of stationary phase entry; external indole is irrelevant in determining the kinetics of stationary phase entry.
2. Advancing stationary phase entry allows external resources to be conserved, allowing long term survival of the cells under starvation conditions.

This chapter will test the validity of these assertions.

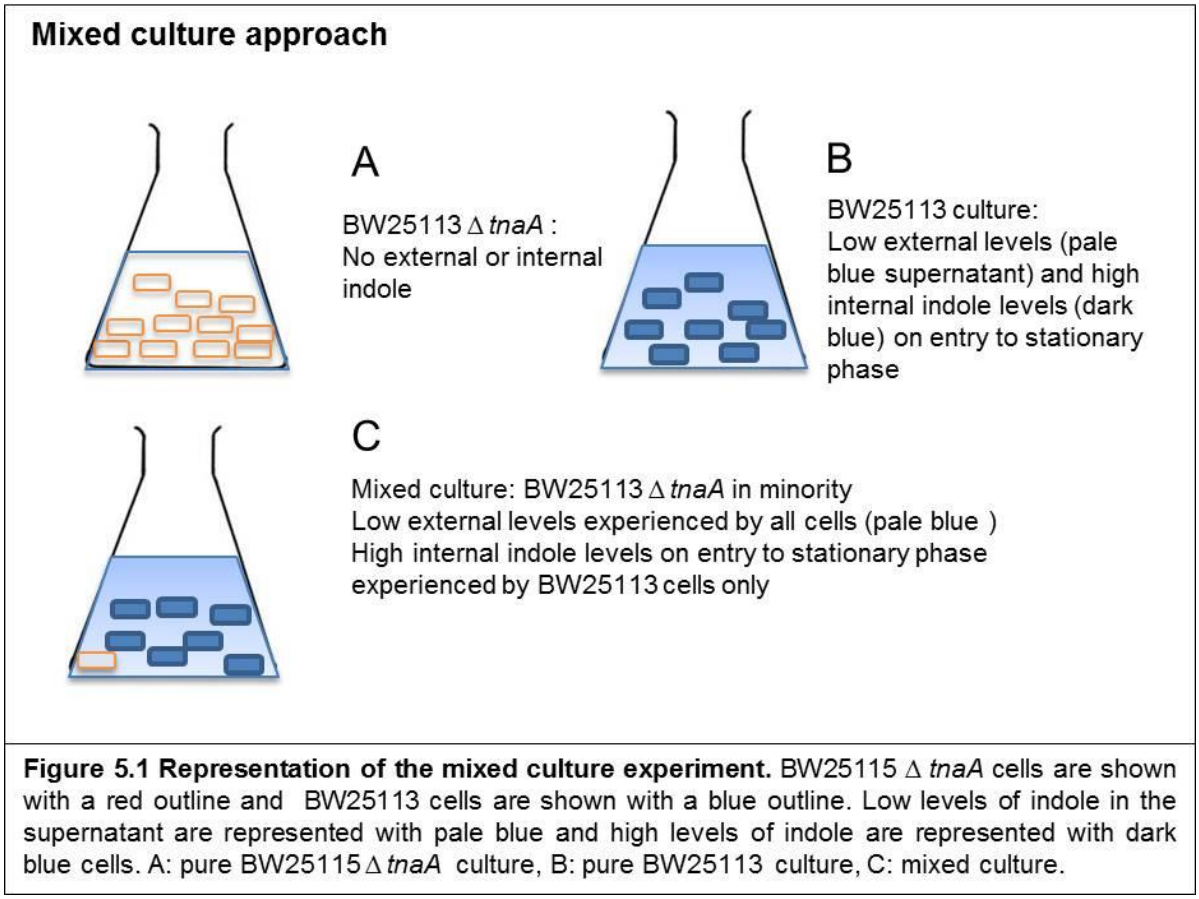


Figure 5.1 Representation of the mixed culture experiment. BW25115 Δ *tnaA* cells are shown with a red outline and BW25113 cells are shown with a blue outline. Low levels of indole in the supernatant are represented with pale blue and high levels of indole are represented with dark blue cells. A: pure BW25115 Δ *tnaA* culture, B: pure BW25113 culture, C: mixed culture.

5.2 An experimental approach to test assertions of the pulse signalling hypothesis

An experimental approach was developed to test these assertions and allow predictions to be made about the outcomes. A mixed culture approach was used, whereby the pulse signalling hypothesis and a knowledge of the behaviour of pure wild-type and pure mutant cultures were used to predict the behaviour of a mixed culture, which contained a known proportion of both wild-type and mutant cells (Fig. 5.1).

In a pure Δ*tnaA* mutant culture, cells do not produce indole on entry into stationary phase (Fig. 5.1A). In a pure wild-type culture, cells experience a low level of external indole throughout stationary phase and a transient high cell associated level of indole during entry into stationary phase (Fig. 5.1B). In a mixed culture all cells in the population will experience the same low external levels of indole in stationary phase. If the mutant cells are present at a sufficiently small proportion the external indole concentration should be indistinguishable from that in a pure wild-type culture (Fig. 5.1C). However, only the wild-type cells in the mixed culture will experience the pulse of high cell associated indole on entry into stationary phase.

Crucially, this provides a way to observe the behaviour of mutant cells that experience low external levels of indole but not the high cell associated levels of indole on entry to stationary phase.

To analyse the mixed culture, the two cell types must be easily distinguishable. The BW25113 Δ *tnaA* Km^R strain can be used which is resistant to kanamycin, while the wild-type BW25113 strain is sensitive. Thus, if samples from the mixed culture are plated onto both LA and LA + kanamycin (30 μg ml^{-1}) only mutant cells will be able to grow on the LA + kanamycin, whereas both mutant and wild-type cells can grow on LA. This allows the proportion of mutant cells in the mixed culture population to be calculated.

Assertion 1 (above) states that the pulse of indole contributes to the timing of stationary phase entry and that low external amounts of indole are irrelevant in stationary phase entry. Fig. 5.2 shows the predicted outcomes of a mixed culture experiment depending upon whether assertion 1 is correct or not.

If assertion 1 is correct (Fig. 5.2A) then in a mixed culture, the mutant cells experience the same low external levels of indole as wild-type but the mutant cells do not experience the pulse of indole, so will enter stationary phase later than the wild-type cells. Therefore the proportion of the mutant cells in the population will increase during entry into stationary phase.

Conversely if assertion 1 is incorrect and the key (Fig. 5.2B), then in a mixed culture, as the wild-type and mutant cells experience the same low external levels of indole, they will enter stationary phase at the same time as the wild-type cells, and their proportion in the population will remain constant.

5.3 In a mixed culture, non indole producing mutants are able to increase in proportion during stationary phase entry.

To test assertion 1, pure wild-type, pure mutant and mixed cultures were set up in parallel. For the pure cultures overnight cultures of BW25113 and BW25113 Δ *tnaA* cells were diluted into fresh LB medium, in triplicate, to an OD_{600} of 0.05. To set up the mixed culture, a 1:100 dilution of BW25113 Δ *tnaA* cells was used to create a initial ratio of 100:1 BW25113 cells to BW25113 Δ *tnaA* cells. These cultures were grown at 37 °C with shaking for 24 hours. The OD_{600} and external indole in the cultures was measured every 2 hours for 8 hours, then at 24 hours (Fig. 5.3).

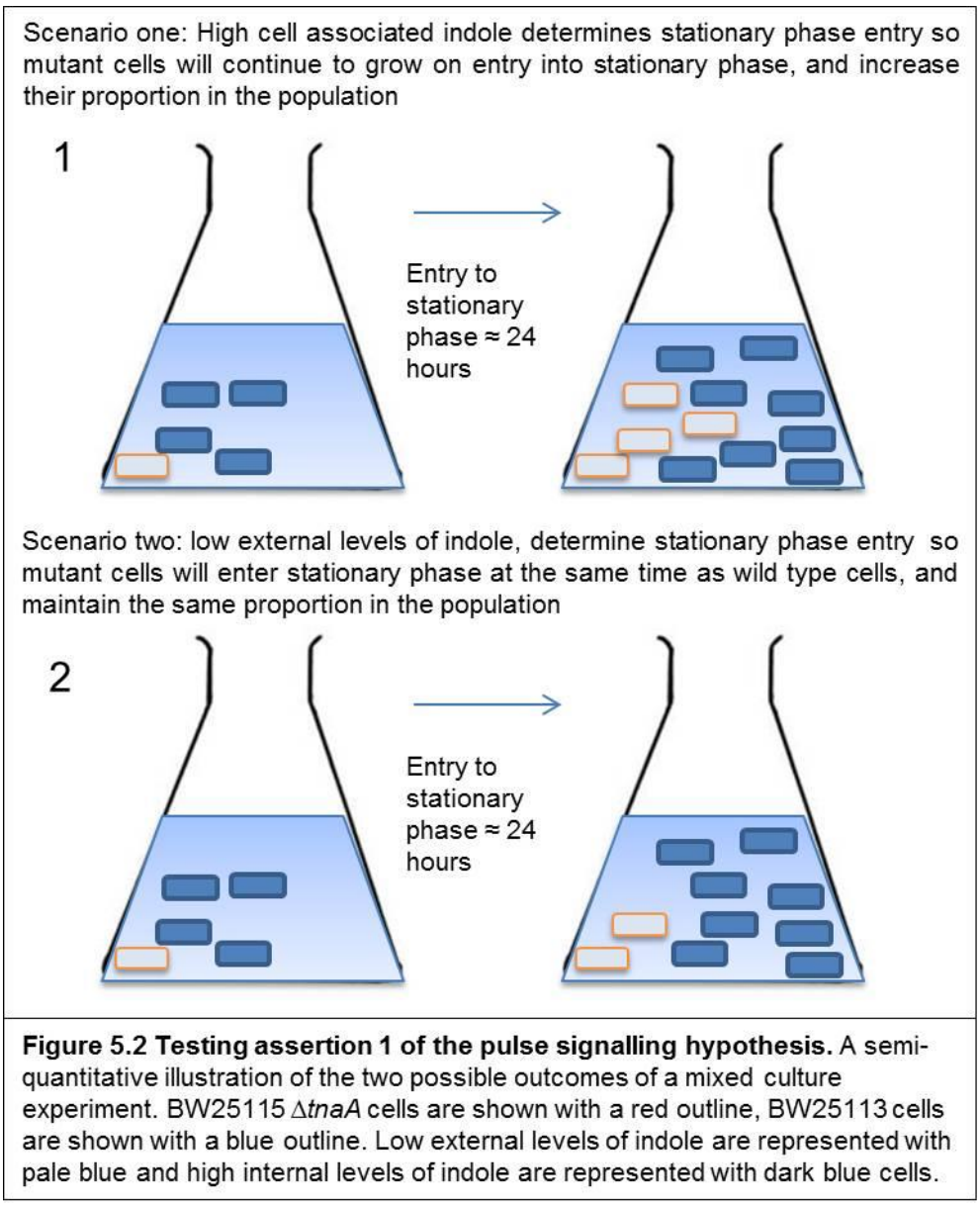
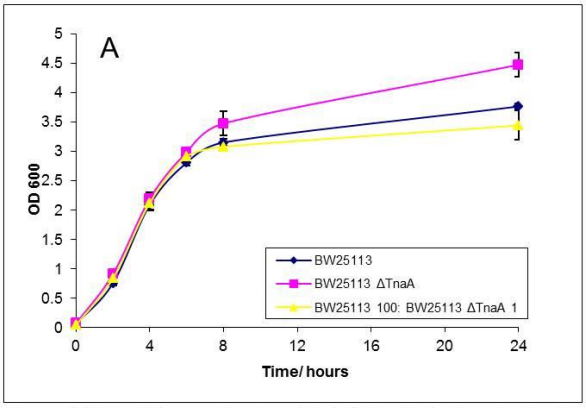


Figure 5.2 Testing assertion 1 of the pulse signalling hypothesis. A semi-quantitative illustration of the two possible outcomes of a mixed culture experiment. BW25115 Δ*tnaA* cells are shown with a red outline, BW25113 cells are shown with a blue outline. Low external levels of indole are represented with pale blue and high internal levels of indole are represented with dark blue cells.

The OD_{600} of the cultures were indistinguishable for the first 6 hours of the experiment (Fig. 5.3A). The first 6 hours represented exponential growth initially followed by slowing of growth on the approach to stationary phase. The rate of increase in OD_{600} in all cultures started to decrease after 6 hours, which corresponded to stationary phase entry. Between 6 and 8 hours the behaviour of the cultures begun to diverge. The mutant culture displayed more slow residual growth than the wild-type or mixed cultures. At 8 hours the OD_{600} of the mutant culture was approximately 3.5, and increased to approximately 4.5 over the next 16 hours. However the wild-type and mixed cultures grew significantly less over this period, with an increase in OD_{600} from approximately 3 to 3.5 over the same period.



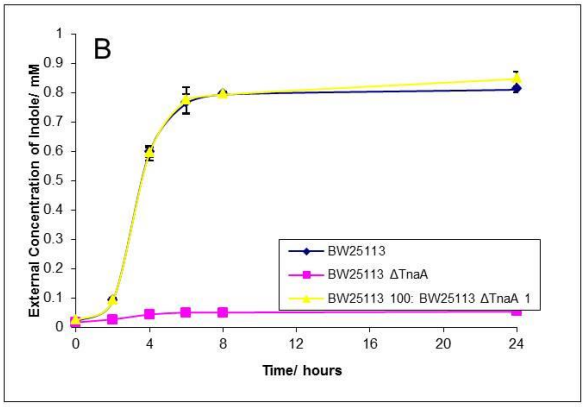


Figure 5.3 The OD $_{600}$ and external indole levels in the mixed culture are the same as a pure BW25113 culture. Samples were removed from pure BW25113, BW25113 Δ *tnaA* and mixed cultures. The OD $_{600}$ (A) was measured and samples were centrifuged to remove cells and the remaining supernatant was assayed for indole using Kovacs assay (B). Data shown are the mean values ± standard deviation for three independent repeats.

The external concentration of indole (Fig. 5.3B) in all cultures was low for the first 2 hours of the experiment. At 4 hours, the external indole in the wild-type and mixed cultures had increased to approximately 0.6 mM. From this we can infer that the pulse of indole occurred between 2 and 4 hours. Following this there was a slight increase in the external indole in the wild-type and mixed cultures, up to approximately 0.8 mM over the next 2 hours. The concentration of indole in these cultures then remained constant. The concentration of indole in the mixed culture was the same as a pure wild-type culture at all times, an important control for this experimental approach. No significant amounts of indole were detectable in the mutant supernatant at any time.

Samples of each culture were diluted appropriately and were plated onto both LA and LA + kanamycin plates. This allowed the proportion of mutant cells in the population to be determined (Table 5.1).

Table 5.1: The proportion of BW25113 Δ *tnaA* cells in mixed culture across at 24 hour period

Proportion of BW25113 Δ *tnaA* in mixed culture [A]	
0hr	0.7±0.4%
2hr	1.1±0.4%
4hr	1.8±0.2%
6hr	1.8±0.4%
8hr	3.3±0.4%
24hr	4.4±0.2%

[A]: . Data shown are the mean values ± standard deviation for three independent repeats.

The proportion of mutant cells in the mixed culture increased from approximately 0.7%, when the cultures were set up, to over 4.4%, after 24 hours. This corresponds to an approximate 6 fold increase in the proportion of mutant cells during the 24 hour period.

The most significant increase in the proportion of mutant cells in the population occurred between 6 and 8 hours. This is also when the OD_{600} of pure wild-type and mutant cultures started to diverge (Fig. 5.3A). This suggests that this increase in the proportion of the mutant cells in the mixed culture at this time is due to the continued growth of the mutant cells at a time when the growth of the wild-type cells is slowing. Furthermore, this corresponded to when indole can be detected in the supernatant at high levels i.e. once the wild-type cells have 'pulsed' (Fig. 5.3B).

This result is consistent with scenario 1 of Fig. 5.2 and suggests that the low external level of indole produced by wild-type cells does not determine the timing of entry of the mutant cells into stationary phase. The only difference between the mutant and the wild-type cells is that the mutant cells do not experience the crucial pulse of indole, so they continue to grow once the wild-type cells have entered stationary phase. This result also explains the observation in Chapter 3, that the

addition of 1 mM external indole is unable to alter the entry of mutant cells into stationary phase (Fig. 3.2).

5.4 In the long term, non indole producing mutants are able to persist at higher proportions in a mixed culture.

Assertion 2 states that the indole pulse results in resources being conserved by wild-type cells, allowing long term maintenance of cell numbers. Fig. 5.4 shows the possible two possible scenarios, depending upon whether the conserved resources are internal or external to the wild-type cells.

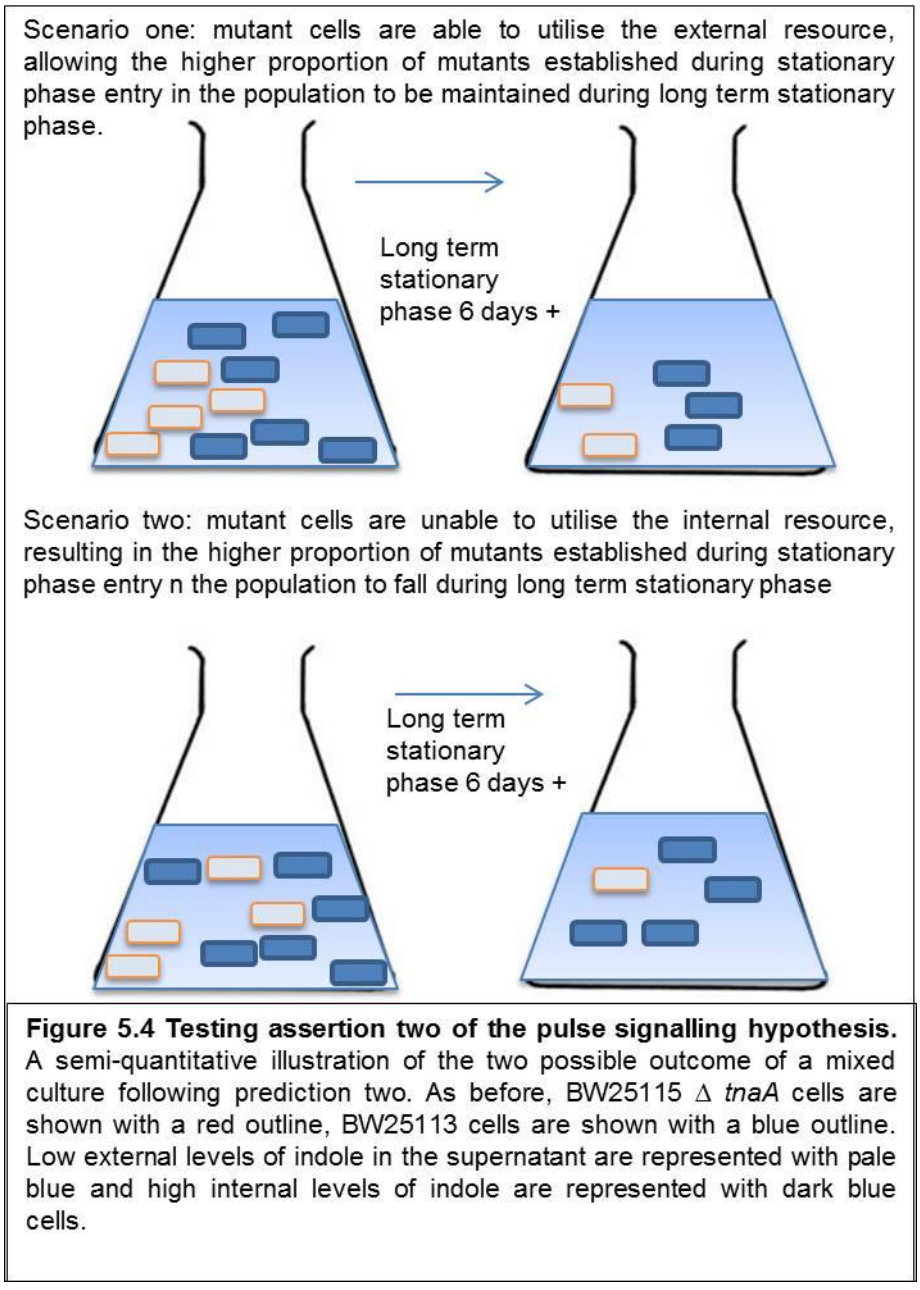


Figure 5.4 Testing assertion two of the pulse signalling hypothesis. A semi-quantitative illustration of the two possible outcome of a mixed culture following prediction two. As before, BW25115 Δ *tnaA* cells are shown with a red outline, BW25113 cells are shown with a blue outline. Low external levels of indole in the supernatant are represented with pale blue and high internal levels of indole are represented with dark blue cells.

In Fig. 5.4 wild-type cells leave a proportion of external resource unused in growth medium during entry into stationary phase. In a mixed culture the mutants will be

able to utilise this resource, resulting in an increased proportion of mutant cells after 24 hours. Therefore, both mutant and wild-type cells will have exactly the same amount of resources available to maintain viability during long term stationary phase, so the proportion of mutant to wild-type cells should not change during this period.

Conversely the pulse may result in wild-type cells storing some resource internally to ensure survival in long term stationary phase. The mutants will not be able to utilise this and the proportion of mutants in the mixed culture will decrease in long term stationary phase.

In order to distinguish between the two scenarios, it was necessary to look at long term density and cell numbers in the cultures. Pure wild-type, pure mutant and mixed cultures were set up in parallel as described previously.

These cultures were grown at 37 °C with shaking for 9 days. Samples were also taken from each culture every 2-3 days and the cell density (by measuring OD $_{600}$) and cell numbers (by diluting appropriately, plating onto LA plates and counting colonies the following day to obtain CFU X 10^6/ ml) were measured (Fig. 5.5).

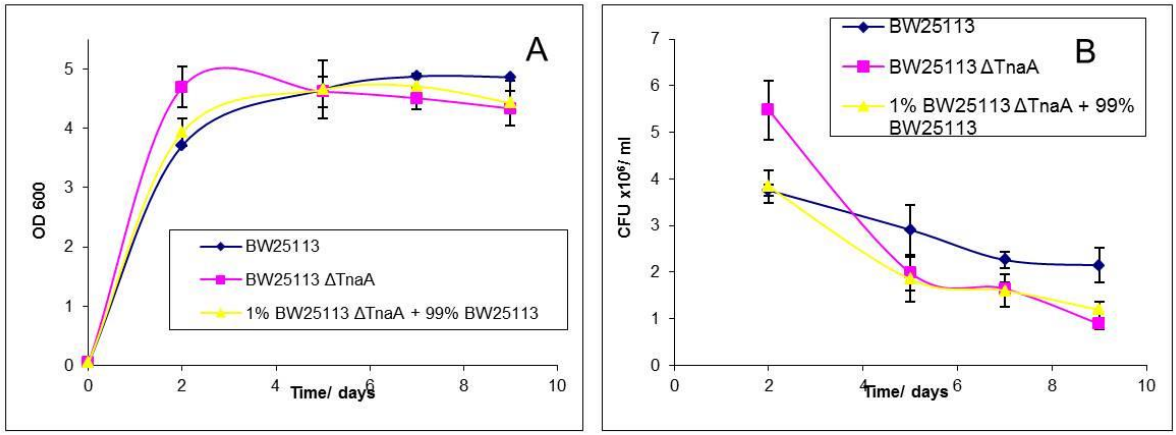


Figure 5.5 The density and cell numbers of a mixed culture initially follows a BW25113 culture, but over the long term tracks the BW25113 Δ *tnaA* culture . The density (OD $_{600}$: A)and cell numbers (CFU x 10^6/ ml :B) of BW25113, BW25113 Δ *tnaA* and a mixed culture were assessed over 10 days. Data shown are the mean values ± standard deviation for three independent repeats

The results show that during the first 5 days the densities of the BW25113 and mixed cultures were significantly lower than the BW25113 Δ*tnaA* cultures, confirming our previous observation. Over the subsequent 4 days the OD $_{600}$ of the mutant and mixed cultures declined, while the density of the wild-type continued slowly to increase. Consequently, after 10 days in stationary phase, it was the wild-

type culture that had the highest density. This trend was also observed in the cell numbers data. Furthermore, across 9 days in stationary phase, the number of cells in the mutant cultures decreased by approximately 80%. However the wild-type culture cell numbers decreased by approximately 40%. This is consistent with the results shown in Fig. 3.2.

The density of the mixed culture was initially the same as the pure wild-type culture, but after 5 days was more like the pure mutant culture. This is presumably because, despite still being in the majority the wild-type cells are unable to survive because the mutant cells have already utilised the resource. This suggests that the resource conserved by the wild-type cells is simply left unused in the growth medium, allowing the mutants to access it, rather than being stored internally.

Table 5.2: The proportion of BW25113 Δ*tnaA* cells in mixed culture across at 9 day period

Proportion of BW25113 Δ *tnaA* in mixed culture [A]	
0days	1.1±0.3%
2days	3.4±0.2%
5days	7.6±0.3%
7days	7.0±0.1%
9days	6.4±0.2%

[A]: . Data shown are the mean values ± standard deviation for three independent repeats.

To monitor changes in the proportion of the mutant cells in the mixed culture samples were diluted appropriately and plated onto both LA and LA + kanamycin plates (table 5.2).The result shows that the proportion of the mutant cells in the mixed culture increased from approximately 1%, when the cultures were set up, to approximately 7%, over the course of 5 days. This is an approximately 7 fold increase in the proportion of mutant cells. However, from 5-9 days the proportion remained at a constant level. This is consistent with scenario one of assertion two, that mutant cells were able to utilise the external resource, allowing the higher proportion of mutants established during stationary phase entry in the population to be maintained during long term stationary phase.

5.5 Discussion

This chapter aimed to test two important assertions made by the pulse signalling hypothesis. The first assertion was that the pulse of indole is a crucial determinant in timing of stationary phase entry, and that low external amounts of indole are unimportant in determining stationary phase entry. This was shown to be correct as in a mixed culture, mutant cells increased their proportion in the culture during the entry to stationary phase, despite experiencing exactly the same amount of external indole, made at the same time and rate as wild-type cells. The only difference between the two cell types in the mixed culture is that the mutant cells do not experience the pulse of cell associated indole. It therefore seems that the subsequent difference between the two cell types was due to the presence or absence of this pulse of indole. Furthermore, this helps explain why, in chapter 3, addition of 1 mM indole is insufficient to restore wild-type behaviour to mutant cells. These indole supplemented cells also do not experience the necessary indole pulse so will continue to display the mutant phenotype, further showing the low external levels of indole are irrelevant here.

The second assertion of the hypothesis was that pulse experienced by wild-type cells causes wild-type cells to enter stationary phase earlier, leading to conservation of resources in the growth medium, which the cells subsequently use to maintain long term viability. This was also shown to be correct, as in a mixed culture, the relative proportions of wild-type and mutant cells did not change throughout long term stationary phase. This suggests that the wild-type cells in the mixed culture conserved resources in the growth medium which the mutant cells are able to access and utilise.

That the resources were available to the mutant cells was a surprising result. If wild-type cells growing in a native environment e.g. the human gut, simply leave external resources in surroundings, it could be imagined that this would be ‘stolen’ by another competing species of bacteria. In the LB growth medium in the experiment described here, it seems that the competing mutant cells can easily access the resources, presumably due to the fact that here the resource is well mixed and dispersed throughout the medium. However in a native environment perhaps competing bacteria may not be able to access the external resource as easily, due to reduced mixing. Perhaps in this case, a local, external store of nutrients could potentially remain available for future use.

Whilst the approach taken was useful in testing the predictions of the hypothesis, it was not without limitations. A BW25113 Δ *tnaA* Km^R mutant strain was used which is resistant to kanamycin, unlike the wild-type BW25113 strain, in order to distinguish the two cell types in a mixed culture. A criticism of this approach is that the two strains no longer only differ in their ability to make indole; the mutant is also kanamycin resistant unlike the wild-type strain. As kanamycin resistance was not being selected for in the cultures, it seems unlikely that the kanamycin resistance will affect density or number of cells in the culture. It is however possible that kanamycin sensitivity/resistance of the strains in the mixed culture had some influence on the result.

To remove this possibility, two derivatives of the ampicillin (Ap) and tetracycline (Tc) resistance plasmid pBR322 (Ap^r, Tc^s and Ap^s, Tc^r) have been created to differentially mark the indole producing and non-producing strains. To check whether any bias is introduced by the marker plasmids, each experiment will be performed twice, with the plasmids switched between strains. This technique has been successfully used to mark cells differentially in the past (Beton *et al.,* 1993).

Preliminary experiments carried out by Antoine Davide (Erasmus summer student, 2013) using this approach have shown similar results to those presented here. Furthermore, this approach was used to look at a mixed culture in which the wild-type cells formed the minority initially (1% BW25113 and 99% BW25113 Δ *tnaA*). No increase in proportion of wild-type cells was observed over a 5 day period, an important control for this experiment, and one which could not be performed using the Km^R strain labelling technique used throughout this chapter.

Alternatively, a fluorescence marker could be used to distinguish the two cell types and the cells sorted by flow cytometery to determine relative proportions.

There remains one key assertion of the hypothesis which has not been thoroughly tested. This is that the pulse of indole directly inhibits growth and cell division on entry into stationary phase. However, various results show that this is highly likely to be the case. First, the result that wild-type cells enter stationary phase at a lower cell density (Fig. 3.2), and that this cannot be induced in the mutant strain by addition of 1 mM indole (Fig. 3.2) or by growing in a mixed culture (this chapter) suggest that the pulse of indole is responsible for slowing growth.

In addition to this, the 60 mM peak of the pulse is equivalent to addition of 4 mM external indole (Fig. 4.3), a level which has previously been show to inhibit growth (Chant and Summers, 2007) and cell division *via* an ionophore based mechanism (Chimerel *et al*., 2012). It therefore seems very likely that the role of the pulse is to slow growth and cell division on entry into stationary phase, resulting in conservation of external resources, described in this chapter.

An important question regarding the nature of the resource left by the wild-type cells remains. The results reported here demonstrate that the resource must be external, as the mutant cells were able to access and utilise it. Future work could address this by measuring concentrations of various nutrients in wild-type and mutant cultures during entry into stationary phase. It would be expected that a wild-type culture will initially (after 24 hours) have more of this resource and that in the long term (+ 6 days) it will become depleted. In the case of the mutant cells, this resource will be depleted earlier.

This chapter aimed to test key aspects of the pulse signalling hypothesis, and the results obtained are consistent with its predictions. The indole pulse may represent a new perspective on indole signalling in *E. coli*, since most previous work regarding indole signalling has focused on persistent exposure to low levels of indole. This example of a “pulse signalling” mechanism expands the repertoire of indole effects on bacteria to include ionophore-mediated effects on cell division and growth.

Subsequent chapters will focus on how cells are able to achieve the pulse of indole by investigating the mechanism behind the upregulation of indole production on entry to stationary phase.

Chapter 6

The mechanism of pulse signalling: the role of RpoS and tryptophanase

6.1 Introduction

Previous chapters have demonstrated that, during entry to stationary phase, *E. coli* strongly upregulates indole production. This results in a transient pulse of indole associated with the cells, which initially slows growth, but improves the long term viability of the culture. This chapter will address the mechanism by which cells increase indole production, during this growth phase transition, resulting in the pulse of indole.

Indole is produced by tryptophanase, which is encoded by the *tnaA* gene. Tryptophanase converts tryptophan into indole, pyruvate and ammonia (Newton and Snell, 1965). The *tnaA* gene forms part of the tryptophanase operon (Deeley and Yanofsky, 1981). As reviewed in the Introduction, expression of the tryptophanase operon is tightly controlled, with mechanisms such as catabolite repression and transcription attenuation playing key roles. Briefly, tryptophanase expression is inhibited by glucose, due to catabolite repression (Wyeth, 1919). Furthermore, when tryptophan levels are low, tryptophanase expression is repressed by premature termination of transcription, whereas when tryptophan levels are high, tryptophanase expression is induced (Yanofsky *et al*., 1991; Gong and Yanofsky, 2002).

When cells are grown in rich medium, indole is detectable in the culture supernatant once cells are in stationary phase (Chant and Summers, 2007, Li and Young, 2012). This is because tryptophanase gene expression is also controlled by the alternative sigma factor, RpoS (Lacour and Landini, 2004). RpoS is known as the stationary phase sigma factor, and directs expression of many genes involved in responses to cellular stress and starvation. Levels of RpoS, and hence tryptophanase, are low during exponential phase of growth but accumulate at the onset of stationary phase.

The role of RpoS in tryptophanase regulation was demonstrated by Lacour and Landini (2004) who performed microarray analysis which showed that in a Δ*rpoS* mutant tryptophanase mRNA expression in stationary phase is decreased 14 fold compared to a wild-type strain. Alongside this Lacour and Landini (2004) also used the indigo assay to monitor indole production. The indigo assay uses styrene monooxygenases expressed from a plasmid, pStyABB (O'Connor and Hartmanns,

1997), to convert indole to indigo. Indigo production can then be measured spectroscopically at 610 nm. They showed that in wild-type cells, indigo and hence indole production increased, in late exponential phase, which corresponded to an OD_{600} of between 1 and 3. Conversely in an Δ*rpoS* mutant, the rate of indole production was much lower throughout late exponential phase and into stationary phase.

These two experiments demonstrate that on entry to stationary phase an increased level of tryptophanase mRNA is observed in an RpoS dependent manner. In addition to this the product of the enzyme catalysed reaction, indole, also increases on entry into stationary phase in an RpoS dependent manner. Together these results allowed the authors to conclude that the reduction in indole production in the Δ*rpoS* mutant was likely due to reduced tryptophanase expression.

Whilst these data on tryptophanase mRNA levels and indole production suggested that tryptophanase protein expression is also controlled by RpoS, the increase in tryptophanase protein levels on entry to stationary phase were not measured directly by Lacour and Landini (2004). Furthermore, although levels of tryptophanase mRNA were compared during exponential growth and once the cells were in stationary phase, events during the growth phase transition were not monitored.

Work presented in previous chapter has shown that the rate of indole production during entry into stationary phase is crucial in producing the pulse of indole and subsequent effects on growth and cell numbers. This chapter will therefore address whether RpoS controlled upregulation of tryptophanase expression is sufficient to explain the kinetics of the indole pulse on entry to stationary phase.

6.2 Increased tryptophanase expression at the onset of stationary phase is dependent on the alternative sigma factor, RpoS.

As previous work only assessed tryptophanase mRNA levels, this chapter will use a fluorescent reporter to monitor tryptophanase protein levels during stationary phase entry.

Two *E. coli* strains (MG1665 TnaA-GFP Km^R and MG1655 TnaA-GFP) were obtained from Gang Li and Kevin Young, Department of Microbiology and Immunology, University of Arkansas. The strains have GFP tagged to

tryptophanase in the normal chromosomal position and under normal expression controls. Briefly, the fusions were constructed by inserting a *GFP::kan* cassette immediately prior to the stop codons of tryptophanase by using λ-Red recombination to produce MG1665 TnaA-GFP Km^R (Datsenko and Wanner, 2000). The *kan* cassette was subsequently removed by using FLP recombinase (Datsenko and Wanner, 2000) to produce MG1655 TnaA-GFP (Li and Young, 2012). These strains can be used to monitor tryptophanase expression, by measuring GFP fluorescence.

In order to also examine the role of RpoS in directing tryptophanase expression, an RpoS null mutant from the *E. coli* stock centre (CGCS), BW25113 Δ*rpoS* was obtained. P1 transduction was used to move the *tnaA-GFP* Km^R insert into wild-type and Δ*rpoS E. coli.* This produced BW25113 *tnaA-GFP* and BW25113 Δ*rpoS tnaA-GFP* respectively.

Overnight cultures of BW25113 *tnaA-GFP*, BW25113 Δ*rpoS tnaA-GFP* and the non GFP tagged parental strains BW25113 and BW25113 Δ*rpoS* were diluted into fresh LB medium, in triplicate, to an OD_{600} of 0.05. These cultures were grown at 37 °C with shaking. Initially, the OD_{600} and fluorescence intensity (480 nm excitation and 510 nm emission) were measured every 60 minutes. After 120 minutes, on the approach to stationary phase, samples were removed every 5 minutes, and the OD_{600} and fluorescence intensity were measured (Fig. 6.1)

The cells grew exponentially for approximately 120 minutes. After this time the growth started to slow significantly, with slow residual growth observed for the remaining 80 minutes. Therefore, the period from 120 minutes onwards corresponds to the transition into stationary phase. There was no difference in the growth of the different strains, indicating neither presence or absence of RpoS and the GFP tag affected growth (Fig. 6.1A).

Initially there was no difference in the fluorescence intensity between the cultures. Given the previous reports of the role of RpoS in the upregulation of *tnaA* transcription, it was surprising during stationary phase entry, the fluorescence values for BW25113 Δ*rpoS tnaA-GFP* culture were higher than BW25113 *tnaA-GFP*

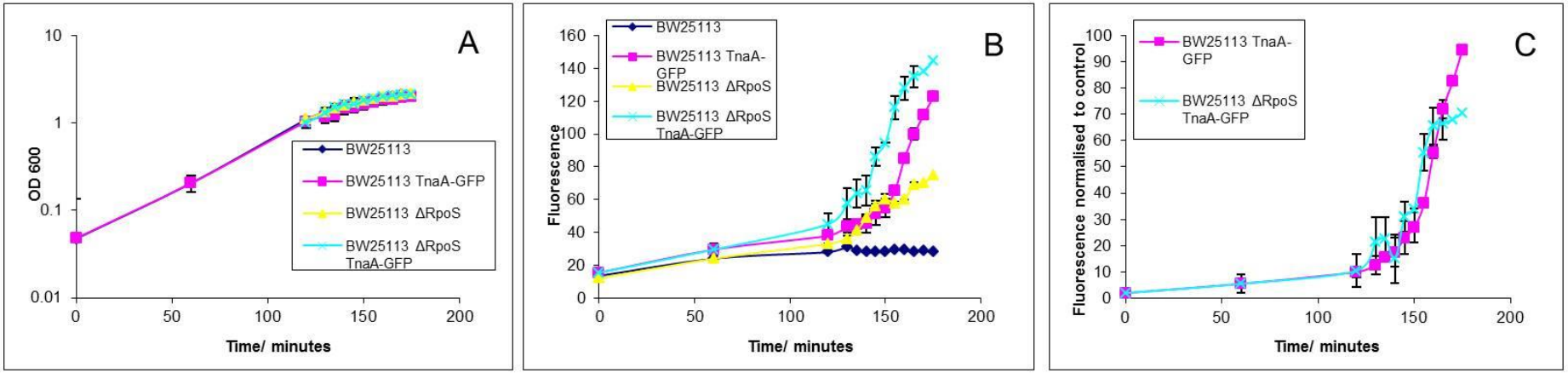


Figure 6.1 Growth and fluorescence data for BW25113, BW25113 Δ *rpoS* , BW25113 Δ *rpoS tnaA-GFP* and BW25113 *tnaA-GFP*. Samples from growing BW25113, BW25113 TnaA-GFP, BW25113 Δ RpoS and BW25113 Δ RpoS TnaA-GFP cultures were removed and the OD_{600} (A) and fluorescence intensity (excitation 480 nm, emission 510 nm) measured (B). The raw data was normalised by subtracting the fluorescence intensity for the non tagged strain for each data point (C). Data shown are the mean values ± standard deviation for three independent repeats

culture at all time points. Unexpectedly, the fluorescence values for the non-tagged BW25113 Δ*rpoS* culture were also significantly higher than the non-tagged BW25113 culture, increasing by approximately 2 fold over the course of the experiment despite the lack of a GFP tag in this strain (Fig. 6.1B).

In order to capture the fluorescence due to *tnaA-GFP* expression, fluorescence values for the tagged strains were normalised to the respective untagged control. The fluorescence value for the GFP tagged strain minus the fluorescence value for the non GFP tagged parental strain was plotted against time (Fig. 6.1C).These data show that initially the normalised fluorescence values for the BW25113 Δ*rpoS tnaA-GFP* culture were similar to the BW25113 *tnaA-GFP* culture. However, there is a point, which corresponds to an OD_{600} of 1-1.5, where the fluorescence intensity normalised to control for the BW25113 *tnaA-GFP* culture, increased above BW25113 Δ*rpoS tnaA-GFP* culture. This continued for the remainder of the experiment. Notably, an OD_{600} of between 1 and 1.5 is typically when indole production is increased, and the pulse is observed (Fig. 4.2)

Using these data, the rate of change of fluorescence per cell for each strain was calculated. The change in fluorescence intensity between two consecutive samples was divided by the change in OD_{600} and the time elapsed.

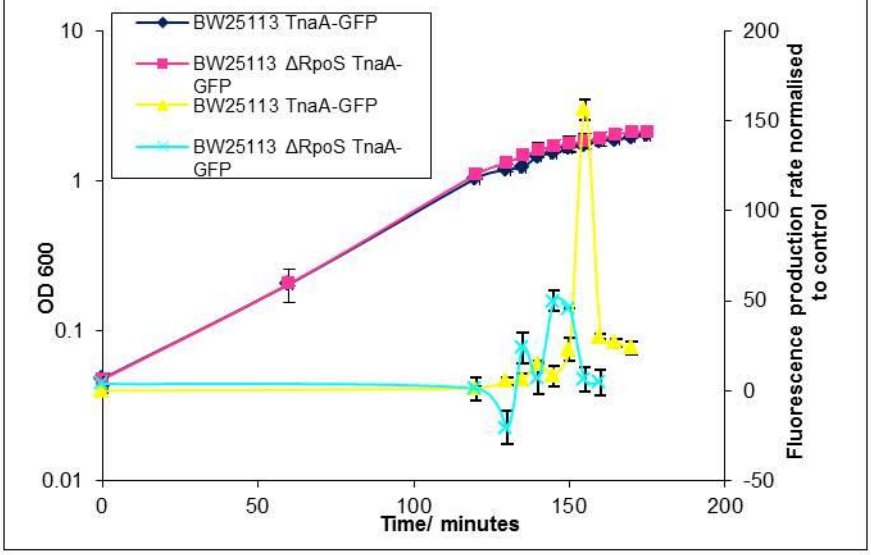


Figure 6.2 BW25113 *tnaA-GFP* fluorescence production rate increases rapidly, at an OD $_{600}$ of 1-1.5, compared to BW25113 Δ *rpoS tnaA-GFP*. Data shown are the OD $_{600}$ values and fluorescence production rate, calculated using the normalised values shown in Fig 6.1 (C).

This variation in the rate of change of fluorescence production over the course of the experiment is shown in Fig. 6.2, alongside the OD $_{600}$ data.

Initially, the fluorescence production rates in the two strains were indistinguishable, at zero. However, when the OD $_{600}$ reached 1-1.5, the fluorescence production rate in the BW25113 *tnaA-GFP* strain increased rapidly, and peaked at a value of approximately 150, before a rapid decrease. The fluorescence production rate of the BW25113 Δ*rpoS tnaA-GFP* strain did not increase as much, and peaked at a lower value of 50, before declining rapidly. The maximum production rate in the BW25113 *tnaA-GFP* strain was approximately 3 times higher than the BW25113 Δ*rpoS tnaA-GFP* strain. This suggests that when RpoS is not present, tryptophanase levels do not increase as quickly at the onset of stationary phase. However, it is clear that this data is not ideal, due to the issues with fluorescence normalisation discussed previously.

6.3 RpoS mutants produce the same amount of indole, but the kinetics of production are altered.

The effect of the altered kinetics of tryptophanase expression in the RpoS mutant on indole production was assessed.

Overnight cultures of BW25113 *tnaA-GFP*, BW25113 Δ*rpoS tnaA-GFP* and the non GFP tagged parental strains BW25113 and BW25113 Δ*rpoS* were diluted into fresh LB medium in triplicate to an OD_{600} of 0.05. These cultures were grown at 37 °C with shaking. The cultures grew exponentially for approximately 120 minutes. After this time the growth started to slow significantly, with slow residual growth observed for the remaining 60 minutes. Therefore, the period from 120 minutes onwards corresponds to the transition into stationary phase. There was no difference in the growth of the different strains, indicating neither presence or absence of RpoS and the GFP tagged affected growth (Fig. 6.3A).

After 120 minutes, on the approach to stationary phase, samples were removed every 5 minutes, and the OD_{600} and concentration of indole in the supernatant were measured using the Kovacs assay (Fig. 6.3B). Initially, ($OD_{600} \approx 1$) in late exponential phase, the concentration of indole in the supernatant in all cultures was low between 0-0.1 mM.

At the onset of stationary phase, ($OD_{600} \approx 1.5$) the external indole concentration of the $RpoS^+$ cultures increased very quickly over a short time (approximately 10 minutes) and reached approximately 0.6 mM. However, although the external indole concentration of the Δ*rpoS* mutant cultures reached the same levels, they did so after a significantly longer time, approximately 25 minutes. Thus, the presence or absence of RpoS affects the kinetics of indole production but not the total amount produced. In addition to this there was no difference in the external indole concentration between the tagged and non-tagged strains, which demonstrates that indole production is not altered by the presence of the tag.

These data were used to calculate the indole production rate per cell for each strain. The change in indole concentration between two consecutive samples was divided by the change in OD_{600} and the time elapsed. This variation in the indole production rate (mM/OD_{600}/ minute) over the course of the experiment is shown in Fig. 6.3C. Initially, the production rates for $RpoS^+$ and Δ*rpoS* mutant cultures were both low. At the onset of stationary phase, the indole production rate transiently peaked at values of approximately 30 in the $RpoS^+$ strain, whereas no increased production rate was observed in the Δ*rpoS* mutant. The production rate in the $RpoS^+$ strain

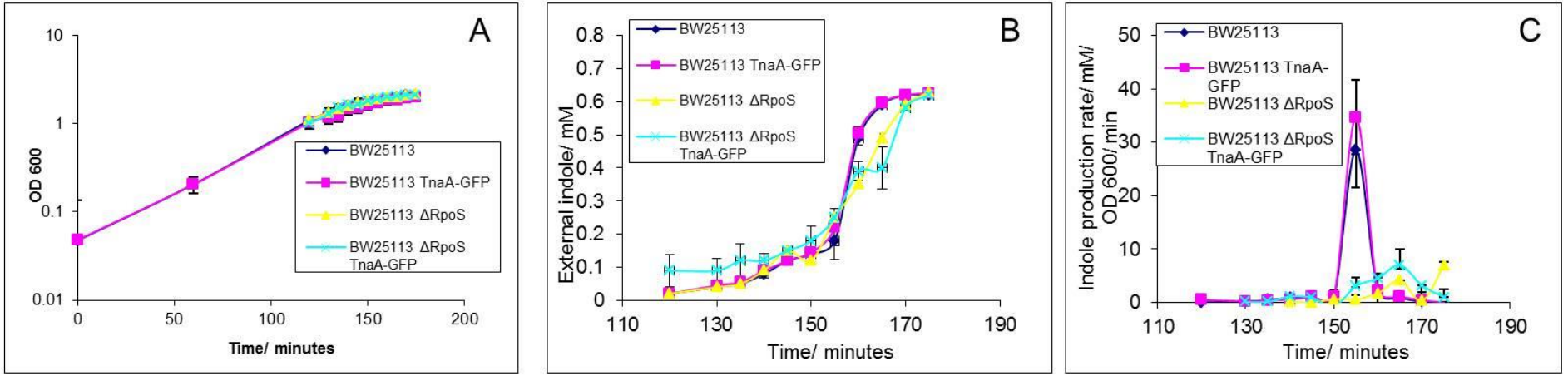


Figure 6.3 External indole in BW25113, BW25113 *tnaA-GFP*, BW25113 Δ*rpoS* and BW25113 Δ*rpoS tnaA-GFP* cells reaches the same levels, but the kinetics of production are altered BW25113 Δ*rpoS* and BW25113 Δ*rpoS tnaA-GFP* cells. Cultures of BW25113, BW25113 *TnaA-GFP*, BW25113 Δ *rpoS* and BW25113 Δ *rpoS TnaA-GFP* cells were sampled regularly. The OD_{600} was measured (A) and samples were centrifuged to remove cells, and the supernatant was assayed for external indole using Kovacs assay (B). Data shown are the mean values ± standard deviation for three independent repeats. These data were used to calculate indole production rate, shown in panel (C).

subsequently fell to zero, whilst the production rate in the Δ*rpoS* mutants remained slightly higher at a value of approximately 5 for the remainder of the experiment.

6.4 RpoS mutants have significantly less cell associated indole on entry into stationary phase.

A prediction of the results presented in Fig. 6.3 is that the maximum level of cell associated indole should be reduced in a Δ*rpoS* mutant, if the pulse is indeed caused by an increased indole production rate.

Overnight cultures of BW25113 *tnaA-GFP*, BW25113 Δ*rpoS tnaA-GFP* and the non GFP tagged parental strains BW25113 and BW25113 Δ*rpoS* were diluted into fresh LB medium in triplicate to an OD_{600} of 0.05. These cultures were grown at 37 °C with shaking. After 120 minutes, on the approach to stationary phase, samples were removed every 5 minutes, and the OD_{600} and cell associated indole concentrations were measured using the Kovacs assay (Fig. 6.4).

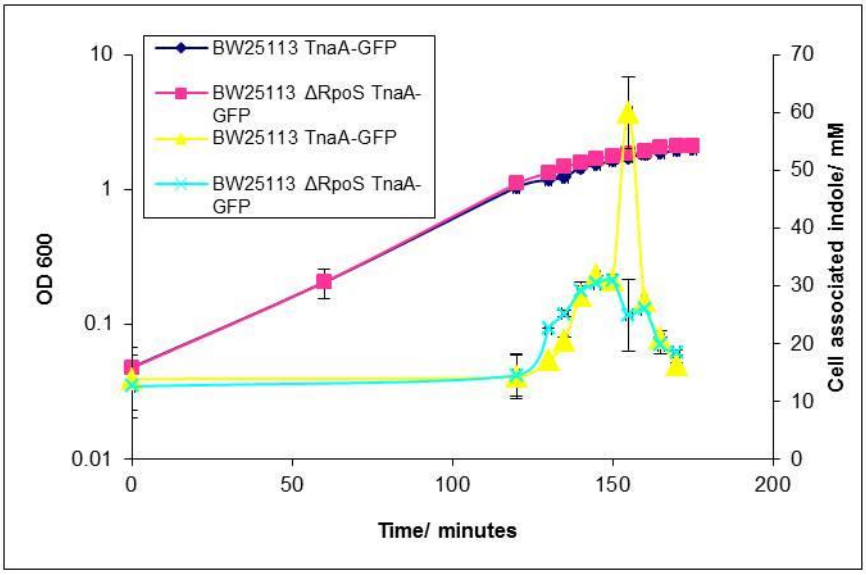


Figure 6.4 Cell associated indole levels are significantly reduced in BW25113 Δ *rpoS tnaA-GFP* cells. Cultures of BW25113 *tnaA- GFP* and BW25113 Δ *rpoS tnaA-GFP* cells were sampled regularly, the OD_{600} was measured and the samples were centrifuged to pellet cells. The resultant cell pellet was assayed for indole using Kovacs assay. Data shown are the mean values ± standard deviation for three independent repeats.

Initially, the cell associated indole for wild-type and Δ*rpoS* mutant cultures were both low at approximately 15 mM. At the onset of stationary phase, at an OD_{600} of approximately 1.5, cell associated indole in both strains increased. The $RpoS^+$

strain peaked transiently at around 60 mM. However, in the ΔrpoS strain there was a significantly lower peak, with a maximum cell associated indole of 30 mM. By the end of the experiment cell associated indole in both cultures was the same at approximately 15 mM.

The results presented in Fig. 6.1-6.4 suggest that in a Δ*rpoS* strain tryptophanase expression does not increase significantly compared to in a wild-type strain. This results in a change in the kinetics of indole production, with no pulse of indole being produced. This helps further demonstrate that the pulse of indole is caused directly as a result of the increased production rate on entry into stationary phase.

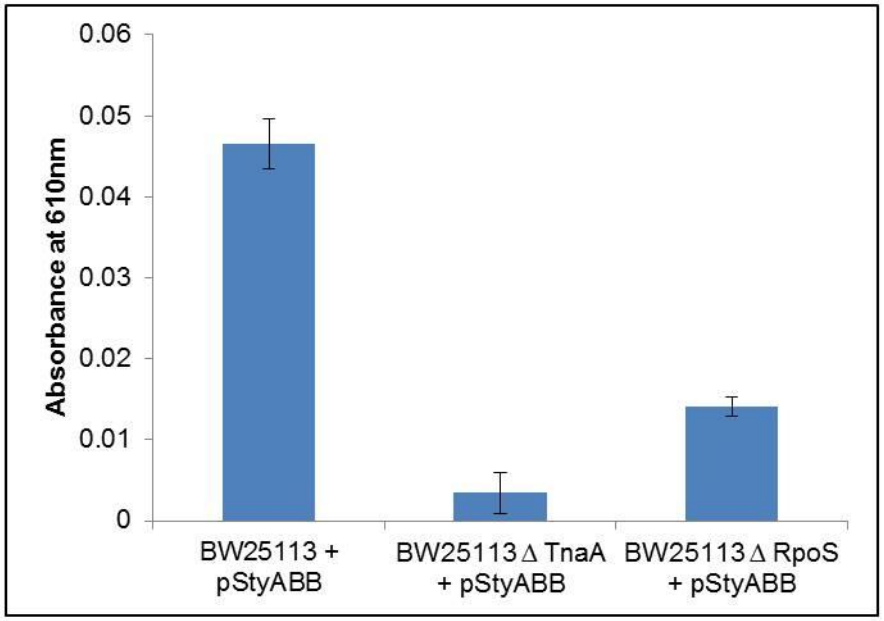


Figure 6.5 Indigo production in BW25113 Δ *tnaA* and BW25113 Δ RpoS cells is significantly reduced compared to BW25113 cells. BW25113, BW25113 Δ *tnaA* and BW25113 Δ *rpoS* cells all containing the pStyABB plasmid were grown for 16 hours. Samples were taken, the cells harvested and subjected to the indigo assay. Data shown are the mean values ± standard deviation for three independent repeats.

Therefore, it is likely that RpoS directed upregulation of tryptophanase is the mechanism by which cells are able to quickly increase indole production on entry into stationary phase. This is consistent with the findings of Lacour and Landini (2004), who showed that the reduction in indole production in the RpoS mutant was correlated with reduced tryptophanase mRNA levels. However, a different technique was used to detect indole in this study.

In order to further confirm the findings detailed in this chapter the indigo assay for indole, as detailed by Lacour and Landini (2004) was used. The indigo assay uses

styrene monooxygenases expressed from a plasmid, pStyABB, to convert indole into indigo. It is therefore specific for intracellular indole. Previous work in this chapter suggests that the maximum level of cell associated indole in a Δ*rpoS* mutant is significantly lower than in $RpoS^+$ cells, so this should be reflected in a reduction in indigo production.

BW25113, BW25113 Δ *rpoS* and BW25113 Δ *tnaA* cells were transformed with the pStyABB plasmid, which encodes styrene monooxygenases. Cultures were grown overnight, samples taken and subjected to the indigo assay (Fig. 6.5).

Indigo production, measured by absorbance at 610 nm, was highest in BW25113 with a value of approximately 0.045. Indigo production in BW25113 Δ *tnaA* (the negative control) was lowest, with a value ≤ 0.005. In the BW25113 Δ*rpoS* strain indigo production was reduced, a value of 0.015 was obtained, which is approximately one third of that produced by the wild-type strain. This is consistent with the findings of Lacour and Landini (2004) who reported indigo production was approximately 3 fold lower in MG1655 Δ*rpoS* than in wild-type MG1655.

6.5 The viability of RpoS mutants is initially similar to wild-type cells, but reduces rapidly, below the levels seen in non indole producing mutants.

In Chapter 3, it was demonstrated that the pulse of indole causes wild-type cells to initially enter stationary phase with a lower density and show increased long term viability. A prediction of the pulse signalling hypothesis would state that if cells do not experience the indole pulse, they enter stationary phase with higher cell density but reduced long term viability.

The results described in this chapter suggest that RpoS mediated upregulation of tryptophanase expression is required for cells to generate the pulse of indole during stationary phase entry. Simplistically, it might be predicted that as the Δ*rpoS* mutant lacks the indole pulse, it might behave like a BW25113 Δ*tnaA* culture. In order to assess the effect of the reduced indole pulse in a Δ*rpoS* mutant the density and cell numbers of a Δ*rpoS* culture were assessed during stationary phase entry and over a longer time period and compared to those seen in wild-type and Δ*tnaA* cultures.

Overnight cultures of BW25113, BW25113 Δ*tnaA* and BW25113 Δ*rpoS* were diluted into fresh LB medium, in triplicate, to an OD_{600} of 0.05. The cultures were grown at 37 °C with shaking. After 24 hours the cell density (OD_{600}) and cell numbers (by

diluting appropriately, plating onto LA plates and counting colonies the following day to obtain CFU X 10^6/ ml) were measured. The cultures were kept shaking at 37 °C for a further 8 days. Samples were taken every 3-4 days and the cell density (and cell numbers were measured (Fig. 6.6).

After 24 hours, the cell density and cell numbers of the Δ*rpoS* mutant culture were similar to the wild-type culture, and both were significantly lower than the Δ*tnaA* culture. However, after 4 days, the cell density and cell numbers of the Δ*rpoS* mutant fell significantly, to lower levels than those seen in the Δ*tnaA* culture. This continued for the remainder of the experiment. This is consistent with previously published work which shows that Δ*rpoS* mutants have significantly reduced viability during prolonged starvation (Lange and Hengge-Aronis, 1991).

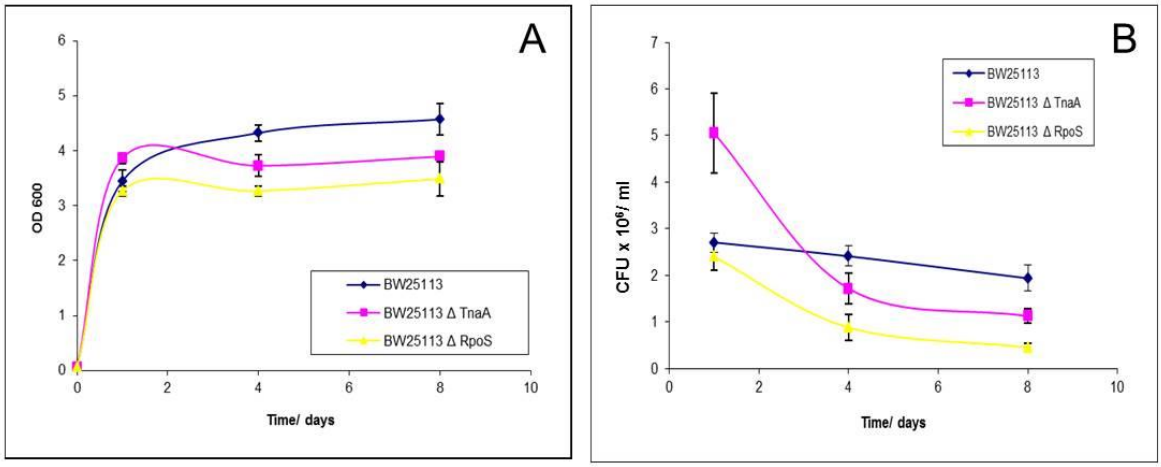


Figure 6.6 The density and cell numbers of BW25113 Δ *rpoS* culture is initially similar to BW25113 but falls rapidly below the levels seen in BW25113 Δ *tnaA* cells over a longer period . The density (OD_{600}: A) and cell numbers (CFU x 10^6/ ml :B) of BW25113, BW25113 Δ *tnaA* and BW25113 Δ rpos cells were assessed over 10 days. Data shown are the mean values ± standard deviation for three independent repeats.

However, this is not consistent with the simple prediction that as the Δ*rpoS* mutant lacks the indole pulse, it should behave like BW25113 Δ*tnaA* throughout the whole experiment. This is perhaps unsurprising as RpoS affects are pleiotropic, with a further 40 genes showing decreased expression in a Δ*rpoS* mutant (Lacour and Landini, 2004). It is however plausible that the decreased indole pulse in the Δ*rpoS* mutant contributes to the reduced density and cell numbers observed in the Δ*rpoS* mutant during extended stationary phase.

6.6 Discussion

This chapter addressed a possible mechanism by which cells might rapidly increase indole production during entry into stationary phase.

Previously it had been shown that indole is detectable when the cells are in stationary phase (Chant and Summers, 2007, Li and Young, 2012). It had also been shown that around the time of stationary phase entry tryptophanase mRNA levels increase significantly in an RpoS-dependent manner (Lacour and Landini, 2004). However, what was not known was if the level of tryptophanase protein also increases at this point, or whether this happens at a point which could provide an explanation for the pulse.

Strains with GFP tagged to tryptophanase allowed the observation of tryptophanase protein levels in wild-type background and Δ*rpoS* mutant backgrounds. In a Δ*rpoS* mutant background, there seemed to be significant reduction in the rate of tryptophanase expression on entry into stationary phase. However, the data seemed somewhat confusing, with a large fluorescence increase also observed for a non GFP tagged strain. Once this was corrected for, it seemed to result in a reduction in the indole production rate in the Δ*rpoS* mutant as was expected. This was also consistent with a significantly reduced indole production rate and pulse of cell associated of indole at the onset of stationary phase. Furthermore, this is consistent with the findings of Lacour and Landini, 2004.

Although this work was not without issues, taken together it seems likely that tryptophanase proteins levels do increase on entry into stationary phase, in an RpoS dependent manner. Furthermore, the work presented here shows that the increase in tryptophanase levels occurs at the same point, during entry to stationary phase, as increased indole production and therefore the pulse, is observed. It therefore seemed plausible that the pulse is caused by the RpoS directed upregulation of tryptophanase, on entry into stationary phase.

Interestingly, despite the fact that indole production rates do not peak at such a high level in an Δ*rpoS* mutant as the wild-type strain, the same final external amount of indole in the Δ*rpoS* mutant is the same as the wild-type strain (Fig. 6.3A). This is important as it gives further support for the pulse signalling hypothesis. It is consistent with the proposal that the pulse of indole occurs due to an increased rate

of production and shows that high transient levels of cell associated indole is important, rather than the external concentration.

The indigo assay was used to confirm the findings described above and the findings of Lacour and Landini (2004). The indigo assay measures internal amounts of indole by conversion of indole into indigo by styrene monooxygenases. This essentially traps the indole inside the cells and the indigo can then be measured spectroscopically. This assay shows a significant reduction in indigo levels in the Δ*rpoS* mutant compared to the wild-type strain, despite the external levels of indole being the same in both strains. Alongside the reduced cell associated indole observed in Fig. 6.4, this helps further show that it is the internal levels of indole which are different in the Δ*rpoS* mutant.

It therefore seemed that the indigo assay could be a potential way to measure levels of indole present in the cytoplasm of cells on entry into stationary phase. It might be expected that the rapid increase in indole production would lead to a rapid increase in indigo production. However, when this was attempted, a rapid increase in indigo production was not observed (data not shown). It seemed that the styrene monooxygenases encoded by the indigo plasmid were unable to efficiently convert the indole pulse into indigo. This seems plausible as the indole pulse happens over a 30 minute period, during which the monooxygenases could have been saturated with substrate. Furthermore, the activities of the styrene monooxygenases are heavily dependent on the availability of oxygen. Oxygen is depleted on entry into stationary phase so this may also have contributed to the failure to detect an increased rate of indigo production in cells approaching stationary phase.

Further problems were that the indigo plasmid, pStyABB, was poorly described in the literature and it seemed unstable. For these reasons, it was decided that the indigo assay was not sufficiently reliable to assay rapid changes in cytoplasmic indole on the entry to stationary phase. It seemed though, that as the results obtained using the indigo assay in this chapter are consistent with those reported by Lacour and Landini (2004) that used in this context the assay is reliable and useful.

Long term cell survival was also measured in the Δ*rpoS* mutant. A simple prediction would suggest that as the Δ*rpoS* mutant lacks the indole pulse it may behave like a Δ*tnaA* strain, both on entry into stationary phase and during prolonged starvation. In fact during stationary phase entry the Δ*rpoS* mutant was most similar to the wild-

type strain, but in the long term was more similar to the Δ*tnaA* strain. Whilst the reduction in long term viability is consistent with the lack of the indole pulse, it is clear that the Δ*rpoS* mutant phenotype is significantly more complex than simply a lack of the indole pulse, as an additional 40 genes show reduced expression in a Δ*rpoS* mutant (Lacour and Landini, 2004). It has previously been suggested that reduced expression of protease genes, *hlsX, lon* and *ClpPX* in an RpoS mutant may contribute to reduced survival of the mutant in long term stationary phase (Dong and Schellhorn, 2009). Therefore, whilst it is possible that the lack of an indole pulse in the Δ*rpoS* mutant may contribute to the reduced density and cell numbers observed in the Δ*rpoS* mutant over an extended period of starvation, it is not possible to assume simple and direct causality.

Whilst these results support the hypothesis that indole production is increased on entry into stationary phase due to RpoS directed expression of tryptophanase, an important question remains regarding the end of the pulse. Here, indole production rate declines rapidly despite tryptophanase still being present (Fig. 6.1) and presumably active. The subsequent chapter will explore the mechanism behind the end of the pulse. Furthermore, the role of glucose as a signal regarding the timing of the pulse will be investigated.

Chapter 7

The mechanism of pulse signalling: the role of tryptophan and glucose

7.1 Introduction

Results described in previous chapters have demonstrated that, on entry into stationary phase, indole production in *E. coli* is upregulated for a brief time. This results in a transient pulse of indole associated with the cells, which persists for approximately 30 minutes. This pulse regulates the timing of stationary phase entry and maximises long term maintenance of cell numbers.

Work presented in the previous chapter was consistent with the idea that increased indole production, and the consequent pulse, are dependent on increased tryptophanase expression during stationary phase entry. It seems likely that tryptophanase expression requires the stationary phase sigma factor, RpoS. It is plausible that on entry into stationary phase, RpoS levels increase, this directs tryptophanase expression and indole production. This sequence of events can help explain how the pulse of indole is initiated.

One remaining question is why the increased indole production rate and the pulse are of short duration. Significantly, although tryptophanase is still present and presumably active, indole production ceases abruptly after approximately 30 minutes. One simple possibility is that indole production stops when available tryptophan is exhausted.

Work performed by Li and Young (2013) demonstrated that the amount of indole produced in *E. coli* is determined directly by the amount of exogenous tryptophan. They reported that when additional tryptophan was provided, cells converted this into equal amounts of indole, up to a maximum concentration of 5 mM. Indeed the concentration of free tryptophan in LB medium has been estimated at between 0.51 mM (Li and Young, 2013) and 1 mM (Sezonov *et al*., 2007). These numbers agree well with the levels of indole measured in the supernatant of stationary phase cultures grown in LB medium, both throughout this thesis and in other published work (Chant and Summers, 2007, Li and Young, 2012).

Addition of extra tryptophan to growing cells can induce indole production in two ways. Firstly, it can induce expression of the *tnaA* operon, resulting in more tryptophanase (Yanofsky *et al*., 1991). Secondly, it simply provides more substrate for tryptophanase to convert into indole.

The aim of the work described in this chapter was to observe the effect of varying the concentration of tryptophan in LB medium on both the total external amount of indole produced and the nature of the indole pulse. In addition, the roles of tryptophan and glucose were further investigated by growing cells in minimal medium, where tryptophan and glucose concentrations could be carefully defined.

7.2 Supplementing LB medium with additional tryptophan increases external indole

In order to assess whether the sudden decrease in indole production at the end of the pulse is caused by the depletion of tryptophan in the growth medium, cells were grown in LB medium containing additional tryptophan.

An overnight culture of BW25113 cells was diluted to an OD_{600} of 0.05 into fresh LB medium supplemented with 0, 1 or 2 mM of additional tryptophan. These cultures were grown at 37 °C with shaking for 370 minutes. Initially, the OD_{600} and concentration of indole in the supernatant were measured once every 60 minutes approximately. After 160 minutes, on the approach to stationary phase, samples were removed every 5 minutes for the next 100 minutes, and the OD_{600} and concentration of indole in the supernatant were measured (Fig. 7.1).

The results show that there was no difference in OD_{600} of the cultures, when 1 or 2 mM additional tryptophan was present in the medium. The cells grew exponentially for approximately 160 minutes. After this, growth started to slow significantly, with slow residual growth observed for the remaining 210 minutes. Therefore, the period from 160 minutes onwards corresponds to the transition into stationary phase for all cultures.

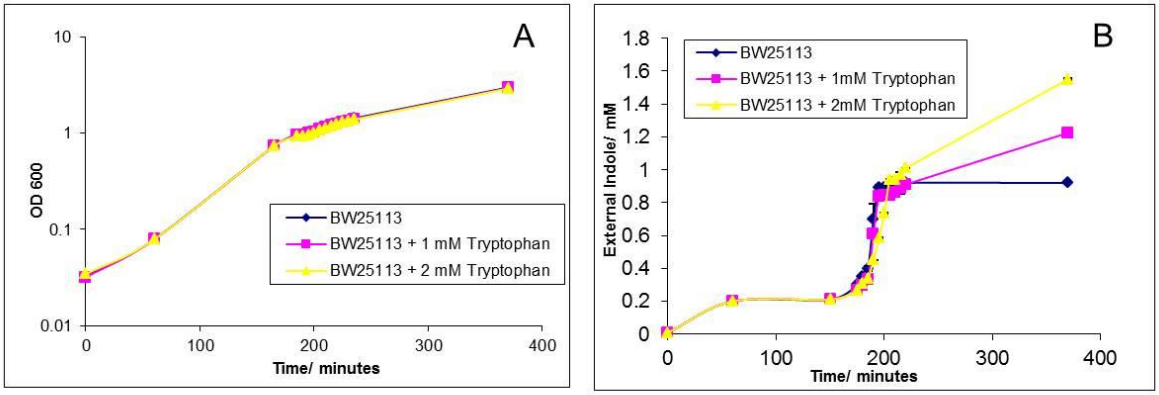


Figure 7.1 Additional tryptophan does not alter growth, but increases the final external indole concentration. A culture of BW25113 in LB medium with 0, 1 and 2 mM tryptophan added was sampled regularly. The OD $_{600}$ was measured (A) and the samples centrifuged to remove cells. The supernatant was assayed for external indole using Kovacs assay (B). Data shown are the mean values ± standard deviation for three independent repeats

The concentration of indole in the supernatant of the three cultures remained low until late exponential phase (approximately 0.2 mM). When the cultures reached an OD $_{600}$ of 1.0-1.5, the concentration rose rapidly to approximately 0.75 mM. This corresponded to the period of transition between exponential growth and much slower growth. In the culture with no additional tryptophan, the external indole reached a maximum concentration of approximately 0.8 mM after 370 minutes. This presumably reflects that conversion of all the tryptophan in the LB medium. In the cultures with additional tryptophan, the external indole continued to increase. At 370 minutes, the culture with 1 mM additional tryptophan had approximately 1.2 mM indole in the supernatant and the culture with 2 mM additional tryptophan had approximately 1.6 mM indole in the supernatant. Thus although additional tryptophan in the medium does not affect growth, or the timing of indole production, the final concentration of indole does increase in response to additional tryptophan.

However, work performed by Li and Young (2013) suggests that all the tryptophan in the media should be converted stoichiometrically into indole eventually. In order to confirm these findings an overnight culture of BW25113 cells was diluted into fresh LB medium, in triplicate, to an OD $_{600}$ of 0.05. These cultures were grown at 37 °C with shaking for 24 hours. At this point the external indole in these stationary phase cultures was assayed and the each culture was split into two.

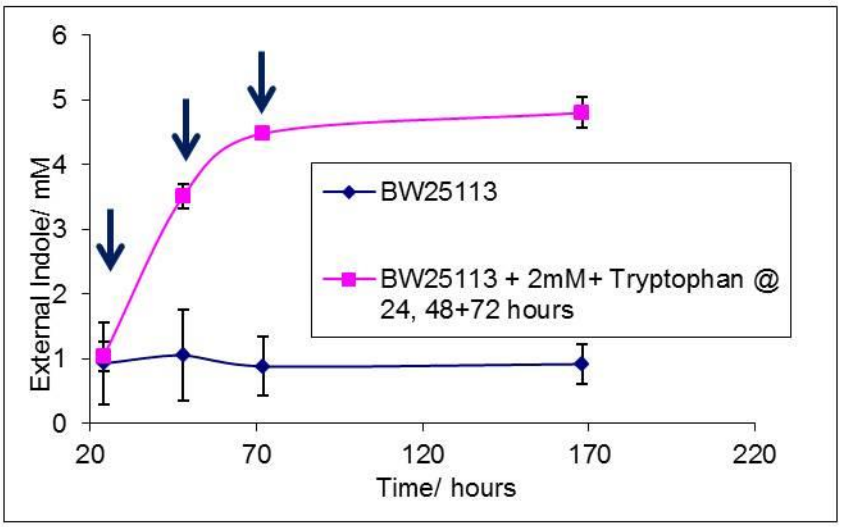


Figure 7.2 Subsequent addition of tryptophan to BW25113 cells increases the external concentration of indole, up to 5 mM. Stationary phase (24 hours) BW25113 cells were incubated with no additional tryptophan or 2 mM tryptophan added every subsequent 24 hours (arrows indicate times of addition). Samples were removed then centrifuged to remove cells. The supernatant was assayed for external indole using Kovacs assay . Data shown are the mean values ± standard deviation for three independent repeats.

One set of cultures continued to be incubated at 37 °C with shaking for a further 144 hours (6 days). In parallel, the other set of cultures was also incubated at 37 °C with shaking for a further 144 hours (6 days). However, 2 mM supplements of tryptophan were added to these cultures at 24, 48 and 72 hours. This resulted in an additional 6 mM tryptophan in these cultures after 72 hours. Samples were taken from the unsupplemented and supplemented cultures every 24 hours for 72 hours then at 168 hours, and the external indole assayed (Fig. 7.2).

At 24 hours, when no tryptophan has been added to either of the cultures, the external indole in all cases was the same at approximately 1 mM. In the unsupplemented cultures, the external indole remained constant at approximately 1 mM for the next 6 days.

In the supplemented cultures, following the addition of 2 mM tryptophan at 24 hours, the external indole had risen to approximately 3 mM by 48 hours. This represents complete conversion of the additional 2 mM tryptophan added to the culture plus the approximate 1 mM present in the LB medium.

Following the addition of a further 2 mM tryptophan at 48 hours, the external indole had risen to approximately 5 mM by 72 hours. Once again, this represents almost

complete conversion of the additional 4 mM tryptophan added to the culture plus the approximate 1 mM present in the LB medium.

Following the addition of a further 2 mM tryptophan at 72 hours, the external indole rose no further and remained at approximately 5 mM even after continued incubation until 168 hours. This is consistent with the findings of Li and Young (2013) who reported conversion of tryptophan into indole stoichiometrically, up to approximately 5 mM.

7.3 Dissecting the role of tryptophan in increased indole production

There are two ways in which tryptophan supplementation can affect indole production. Most simply having more substrate (tryptophan) for the existing tryptophanase should result in more product (indole). Also, the additional tryptophan could increase tryptophanase expression. In this case the amount of enzyme would increase as well as the availability of substrate.

As reviewed in the introduction, tryptophanase expression is induced in the presence of tryptophan. However, it is not known whether significant tryptophanase induction occurs, in response to additional tryptophan, at the onset to stationary phase i.e. at the time of the indole pulse. In order to test this, the effect of exogenous tryptophan on tryptophanase expression was measured during stationary phase entry.

Tryptophanase expression was estimated from the fluorescence of a strain expressing GFP-tagged tryptophanase. This work was performed prior to the construction of BW25113 *tnaA-GFP* used in Chapter 6. Therefore, tryptophanase expression was measured in the original MG1655 TnaA-GFP strain, with the parental MG1655 strain used as the control. A control experiment showed that neither the growth (measured by OD_{600}) or indole production (measured by assaying external indole concentrations) in these strains was significantly different from BW25113 cells (Fig. 7.3)

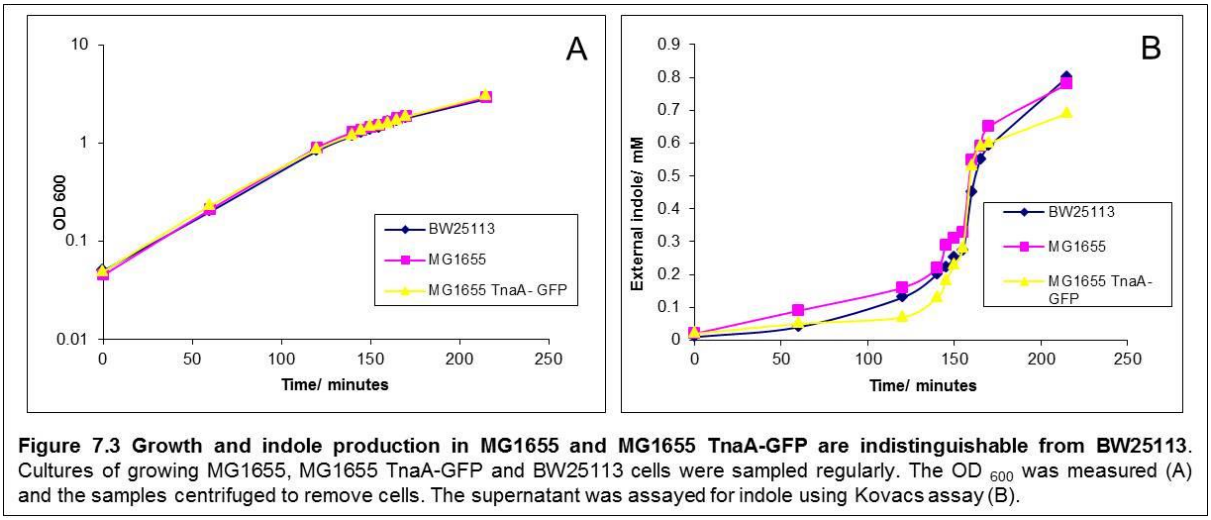


Figure 7.3 Growth and indole production in MG1655 and MG1655 TnaA-GFP are indistinguishable from BW25113. Cultures of growing MG1655, MG1655 TnaA-GFP and BW25113 cells were sampled regularly. The OD $_{600}$ was measured (A) and the samples centrifuged to remove cells. The supernatant was assayed for indole using Kovacs assay (B).

7.4 During entry into stationary phase, no additional tryptophanase expression is observed when the cultures are supplemented with tryptophan.

An overnight culture of MG1665 TnaA-GFP was diluted in triplicate into fresh LB medium containing 0, 0.5, 1 or 2 mM additional tryptophan to an OD $_{600}$ of 0.05. A sample was taken immediately and the fluorescence intensity measured. These cultures were incubated at 37 °C with shaking for 120 minutes. This took the cells to the point where growth slows, the transition to stationary phase begins and indole production starts. A sample was taken and the fluorescence intensity measured to see if there was any difference in tryptophanase expression, in response to increased tryptophan availability (Fig. 7.4).

The result shows that before the addition of tryptophan, all cultures displayed the same fluorescence intensity, with values typically between 5-10. After the addition of tryptophan and incubation at 37 °C with shaking for 120 minutes, the fluorescence values in all cases had increased by approximately 3-4 fold to between 25-35. There was no difference depending on the tryptophan concentrations in the cultures. Therefore it seems that adding extra tryptophan (beyond what is normally present in LB medium) had no significant effect on tryptophanase expression during entry into stationary phase. This is consistent with data presented by Li and Young (2013) who showed that there was no tryptophan dependent effect on TnaA-GFP fluorescence intensity and therefore tryptophanase expression, until after 3 hours of growth, which corresponded to the transition into stationary phase.

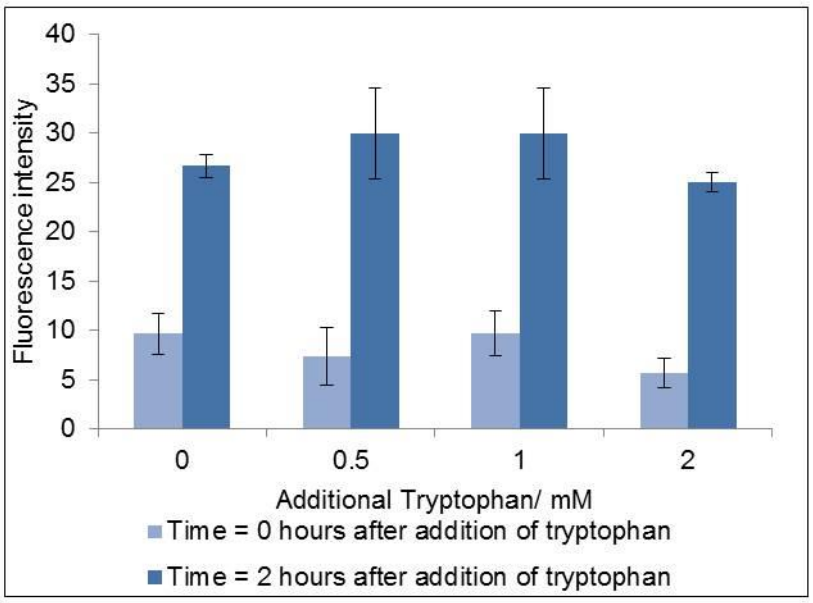


Figure 7.4 Fluorescence intensity of MG1655 TnaA-GFP is not altered by the addition of tryptophan 2 hours after addition. A culture of MG1655 TnaA-GFP cells in LB medium were spilt and supplemented with 0, 0.5, 1, or 2 mM tryptophan. The fluorescence intensity (excitation 480 nm, emission 510 nm) of samples was measured immediately and after 2 hours further incubation at 37 °C with shaking (the approximate time of the indole 'pulse'). Data shown are the mean values ± standard deviation for three independent repeats.

However, Li and Young (2013) also showed that over a longer time period, tryptophanase expression was shown to be dependent on concentration of tryptophan added. To confirm this result an overnight culture of MG1665 TnaA-GFP was diluted in triplicate into fresh LB medium containing 0, 0.5, 1 or 2 mM additional tryptophan to an OD_{600} of 0.05. The cultures were grown at 37 °C with shaking for approximately 16 hours. After 16 hours, when the cells were in stationary phase, samples were taken and the fluorescence intensity measured (Fig. 7.5). The result shows that, after 16 hours, higher concentrations of tryptophan resulted in increased expression of tryptophanase. This is consistent with data presented by Li and Young (2013), who showed that there is tryptophan concentration dependence on fluorescence intensity and therefore tryptophanase expression, after 10 hours of incubation, when the cells are in stationary phase.

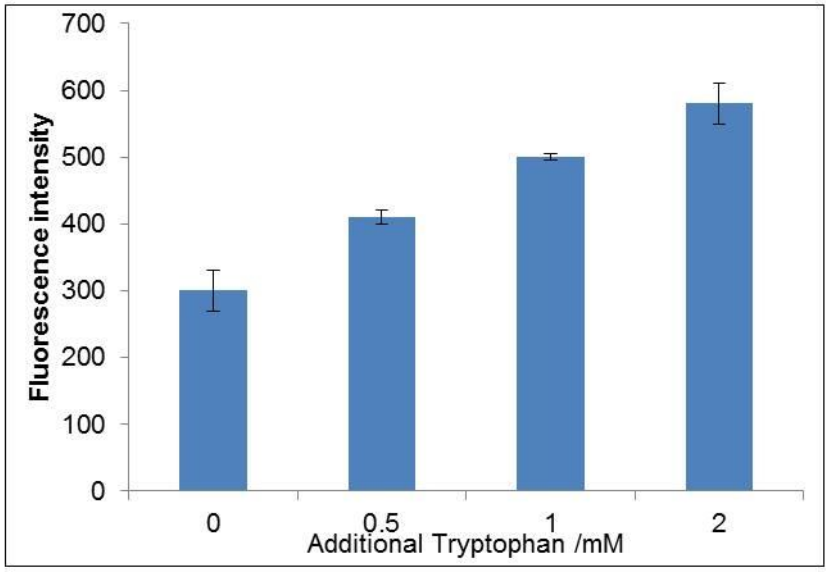


Figure 7.5 Fluorescence intensity of MG1655 TnaA-GFP is increased 16 hours after addition of 0.5, 1, 2 mM tryptophan. A culture of MG1655 TnaA-GFP cells in LB medium was spilt and tryptophan added. The fluorescence intensity (excitation 480 nm, emission 510 nm) of samples was measured after 16 hours. Data shown are the mean values ± standard deviation for three independent repeats.

7.5 Supplementation of LB medium with additional tryptophan causes increased amounts of cell associated indole

Previous results have shown that additional tryptophan in the growth medium is converted quantitatively into indole up to maximum of approximately 5 mM. However, these experiments only measured the external indole produced in response to additional tryptophan. It is also important to assess the effect of additional tryptophan on cell associated indole.

An overnight culture of BW25113 cells was diluted in triplicate into fresh LB medium to an OD_{600} of 0.05. These cultures were grown at 37 °C with shaking for 270 minutes. After 130 minutes, on the approach to stationary phase, each cultures was divided into two split, one had 0.5 mM additional tryptophan added while the other culture did not. Samples were removed every 5 minutes for the next 45 minutes, then every 10 minutes for the remainder of the experiment. The OD_{600}, the concentration of indole in the supernatant and the cell associated indole in the samples were measured (Fig. 7.6).

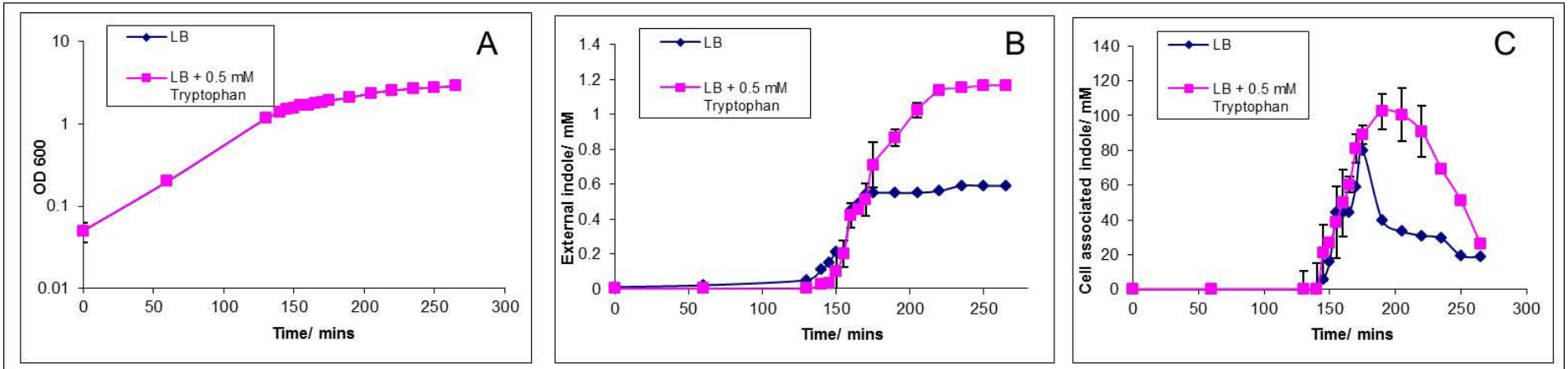


Figure 7.6 Addition of 0.5 mM tryptophan to BW25113 cells growing in LB medium does not affect growth, but increases external and cell associated indole. A culture of growing BW25113 in LB medium with 0 or 0.5 mM tryptophan added was sampled regularly. The OD_{600} was measured (A) and the samples centrifuged to pellet cells. The supernatant was assayed for external indole using Kovacs assay (B) and the cell pellet was assayed for indole using Kovacs assay (C). Data shown are the mean values ± standard deviation for three independent repeats.

There was no difference in growth between the cultures, with or without 0.5 mM additional tryptophan present in the medium. The cells grew exponentially for approximately 130 minutes. After this time, growth started to slow significantly, with slow residual growth observed for the remaining 260 minutes. Therefore, the period from 130 minutes corresponds to the transition into stationary phase.

The concentration of indole in the supernatant of all cultures was low (below 0.2 mM) in late exponential phase. When the culture reached an OD_{600} of 1.0-1.5, the concentration in all cultures rose rapidly to approximately 0.6 mM. This corresponded to the period of transition between exponential growth and much slower growth.

In the cultures with no additional tryptophan, the external indole concentration stopped increasing at approximately 0.6 mM. This reflects the conversion of all tryptophan available in the LB medium into indole. In the cultures with 0.5 mM additional tryptophan, the external indole continued to increase for a further 50 minutes, eventually reaching a concentration of approximately 1.1 mM. This presumably reflects the conversion of all the tryptophan available in the LB medium (0.6 mM) plus the 0.5 mM supplement into indole.

In the cultures with no additional tryptophan added the cell-associated indole began to increase quickly at an OD_{600} of approximately 1.5. This increase in cell associated indole was observed for approximately 30 minutes, reaching a peak of approximately 75 mM. The cell associated indole decreased quickly at first, and then more gradually, to approximately 15 mM.

In the cultures with 0.5 mM additional tryptophan the cell-associated indole also began to increase quickly at an OD_{600} of approximately 1.5. This increase continued for approximately 45 minutes, reaching a peak of approximately 100 mM. The cell associated indole then decreased much more slowly than the culture without additional tryptophan. However it eventually reached approximately 15 mM. Thus the effect of tryptophan supplementation was to make the pulse both higher and wider than in unsupplemented LB medium.

As shown previously (Fig. 7.4), this increase in indole production is not due to increased tryptophanase expression. Addition of 0.5 mM tryptophan causes no

increase in tryptophanase expression across this period. Therefore, the increased indole production is caused simply by the presence of extra substrate for tryptophanase.

7.6 When cells are grown in minimal medium without tryptophan present, no indole is produced.

For a complete analysis between the concentration of tryptophan and indole production, it is important to measure indole production when no external tryptophan is present. This was done by growing cells in minimal medium, which contained no tryptophan. Overnight cultures of MG1655 TnaA-GFP and MG1655 were diluted in triplicate into fresh minimal medium + 0.4% glucose to an OD_{600} of 0.05. These cultures were grown at 37 °C with shaking for 10 days. Samples were removed every 24 hours. The concentration of indole in the supernatant and cell associated indole were measured. There was no detectable external indole or cell associated indole in any cultures at any time (data not shown), suggesting that in the absence of external tryptophan in the growth medium, the internal pool of tryptophan is not converted into indole. This is consistent with the findings from Li and Young (2013).

7.7 When cells are grown in minimal medium with added tryptophan, indole is produced.

In order to observe the effect of exogenous tryptophan concentrations on indole production cells were grown in minimal medium supplemented with 0.5 mM tryptophan.

Overnight cultures of MG1655 TnaA-GFP and MG1655 cells were diluted in triplicate into fresh minimal medium + 0.4% glucose, with or without 0.5 mM tryptophan to an OD_{600} of 0.3. These cultures were grown at 37 °C with shaking for 24 hours. Samples were removed every hour for 8 hours, then after 24 hours. The OD_{600}, fluorescence intensity and the concentration of indole in the supernatant were measured (Fig. 7.7).

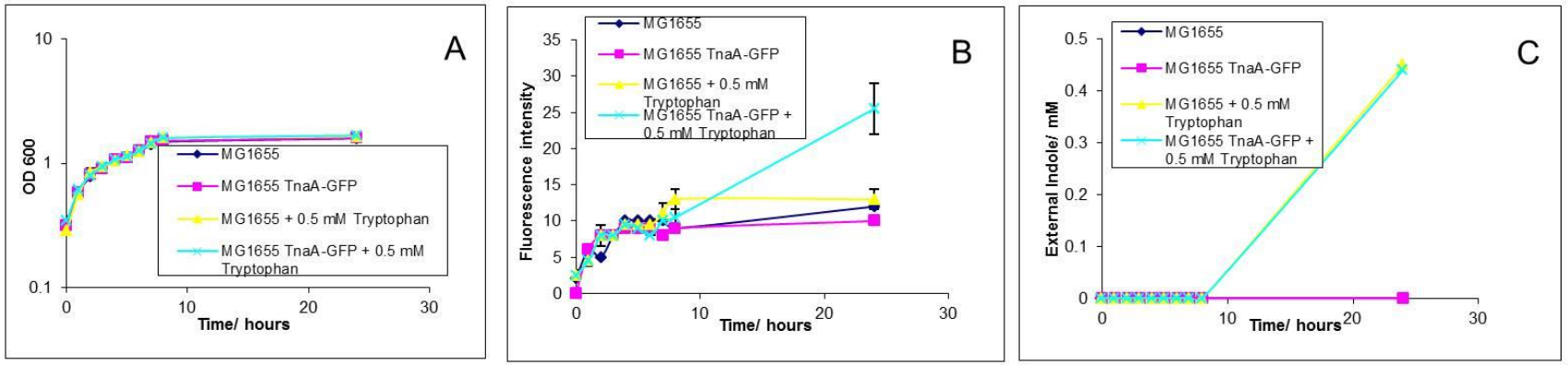


Figure 7.7 MG1655 and MG1655 TnaA-GFP grown in minimal medium with 0.4% glucose produce TnaA and indole when 0.5 mM tryptophan is added and the cells are in stationary phase. MG1655 and MG1655 TnaA- GFP cells were grown in minimal medium with 0.4% glucose, with and without 0.5 mM tryptophan. Samples were removed hourly for 8 hours then at 24 hours. The OD_{600} (A) and fluorescence intensity (excitation 480, emission 510 nm) (B) were measured . The samples were centrifuged to remove cells. The supernatant was assayed for external indole using Kovacs assay (C). Data shown are the mean values ± standard deviation for three independent repeats.

All cultures grew over 8 hours increasing from an OD_{600} of approximately 0.3 to 1.5. There was no further growth of the cultures between 8 and 24 hours. The fluorescence intensity of all cultures increased over the first 8 hours of the experiment, from approximately 4 to 9-14. This reflects the increase in cell density rather than an actual increase in fluorescence per cell. Furthermore, this was not GFP dependent fluorescence, as all the cultures, including the non-GFP tagged controls displayed this increase. After 24 hours, the fluorescence of the MG1655, MG1655 TnaA-GFP and MG1655 + 0.5 mM tryptophan cultures had stayed constant at approximately 9-14. The fluorescence of the MG1655 TnaA-GFP + 0.5 mM tryptophan culture had increased to approximately 25. This does not represent an increase in cell numbers, but is a clear increase in fluorescence per cell. This reflects the tryptophan dependent induction of tryptophanase, since no increase was observed in for a culture of the same strain in the absence of tryptophan.

No external indole was detected in any of the cultures for the first 8 hours of the experiment. After 24 hours, no indole was detected in the supernatants of MG1655 or MG1655 TnaA-GFP cultures lacking tryptophan in the medium. However, after 24 hours, approximately 0.45 mM indole was detected in the supernatant of MG1655 + 0.5 mM tryptophan and MG1655 TnaA-GFP + 0.5 mM tryptophan cultures. Indole was produced by these cultures as tryptophanase had been expressed at this time and tryptophan was present in the growth medium.

In summary, when grown in minimal medium + 0.4% glucose, tryptophanase is only expressed and indole is only made, when the cells are in stationary phase. This is strikingly different from the result in LB medium, where tryptophanase and therefore indole are produced during the transition from exponential phase to stationary phase.

7.8 Glucose regulates the timing of indole production

The expression of tryptophanase has been shown to be RpoS dependent (Lacour and Landini, 2004). In turn, RpoS levels are controlled by a number of factors (reviewed in the Introduction), including glucose availability (For review, see Peterson *et al.,* 2005). Furthermore, expression of tryptophanase also directly is controlled by catabolite repression (Wyeth, 1919). The experiments described in section 7.7 used minimal medium supplemented with 0.4% glucose. It seemed likely that tryptophanase expression and indole production were not observed until the

cells were in stationary phase, because glucose was not limiting until this point. Therefore, the experiment was repeated using a range of glucose concentrations, to see how this affected tryptophanase expression and indole production.

Overnight cultures of MG1655 TnaA-GFP and MG1655 were diluted into fresh minimal medium + 0.2%, 0.1 % or 0.05% glucose in triplicate, with or without 0.5 mM tryptophan to an OD_{600} of 0.5. These cultures were grown at 37 °C with shaking for 24 hours. Samples were removed every hour for 8 hours, then again after 24 hours. The OD_{600}, fluorescence intensity and the concentration of indole in the supernatant were measured for each sample (Fig. 7.8).

When the cells were grown in the presence of 0.2% glucose, the results were very similar to those observed when cells are grown in the presence of 0.4% glucose. All cultures grew over 8 hours increasing from an OD_{600} of approximately 0.5 to 1.5. Growth of all cultures had completely stopped by 24 hours. The fluorescence intensity of all cultures increased over 8 hours, from approximately 4 to 12. After 24 hours, the fluorescence of the MG1655, MG1655 TnaA-GFP and MG1655 + 0.5 mM tryptophan cultures stayed constant at approximately 12 while the fluorescence of the MG1655 TnaA-GFP + 0.5 mM tryptophan culture had increased to approximately 25. No external indole was detected in any culture for the first 8 hours of the experiment. After 24 hours, no indole was detected in the supernatant of MG1655 or MG1655 TnaA-GFP cultures but approximately 0.45 mM indole was detected in the supernatant of MG1655 + 0.5 mM tryptophan and MG1655 TnaA-GFP + 0.5 mM tryptophan cultures. A plausible explanation of this results could be that when the cells are grown in the presence of 0.2% (or 0.4%) glucose, glucose becomes limiting at some point between 8 and 24 hours. This then triggers RpoS expression, which in turn allows tryptophanase expression and indole production as observed. Alternatively, when glucose becomes limiting, catabolite repression of the tryptophanase operon is prevented, allowing tryptophanase expression. If these are correct reducing the glucose concentration in the medium should advance the time of indole production.

When the cells were grown in the presence of 0.1% glucose (Fig. 7.8, 2nd row), cells enter stationary phase much earlier than in the presence of higher glucose concentration. Growth of all cultures stopped after 6 hours, at an OD_{600} of approximately 1.2. This result suggests that, when grown in the presence of 0.1% glucose, glucose becomes limiting after approximately 6 hours of growth, causing

the cells to stop growing and enter stationary phase. When the cells are grown in the presence of 0.05% glucose (Fig. 7.8, 3rd row), cells enter stationary phase earlier again, growth of all cultures had stopped after 5 hours, at an OD_{600} of approximately 0.9. This result suggests that, when grown in the presence of 0.05% glucose, glucose becomes limiting after approximately 5 hours of growth, causing the cells to stop growing and enter stationary phase.

In the presence of 0.1% glucose, fluorescence intensity of the MG1655, MG1655 TnaA-GFP and MG1655 + 0.5 mM tryptophan cultures increased over 24 hours from approximately 4 to 12. The fluorescence of the MG1655 TnaA-GFP + 0.5 mM tryptophan culture had increased to approximately 25 after 6 hours. It continued to increase slowly for the remainder of the experiment, reaching a value of approximately 35 after 24 hours. In the presence of 0.05% glucose, fluorescence intensity of the MG1655, MG1655 TnaA-GFP and MG1655 + 0.5 mM tryptophan cultures increased over 24 hours from approximately 4 to 12. The fluorescence of the MG1655 TnaA-GFP + 0.5 mM tryptophan culture had increased to approximately 25 after 5 hours. It continued to increase slowly for the remainder of the experiment, reaching a value of approximately 45 after 24 hours

In the presence of 0.1% glucose, no external indole was detected in any cultures for the first 6 hours of the experiment. After 24 hours, no indole was detected in the supernatant of MG1655 or MG1655 TnaA-GFP cultures but after 6 hours, approximately 0.45 mM indole was detected in the supernatant of MG1655 + 0.5 mM tryptophan and MG1655 TnaA-GFP + 0.5 mM tryptophan cultures. The indole continued to increase slowly until a maximum value of approximately 0.5 mM was reached after 7 hours. In the presence of 0.05% glucose no external indole was detected in any cultures for the first 4 hours of the experiment. After 24 hours, no indole was detected in the supernatant of MG1655 or MG1655 TnaA-GFP cultures but after 5 hours, approximately 0.45 mM indole was detected in the supernatant of MG1655 + 0.5 mM tryptophan and MG1655 TnaA-GFP + 0.5 mM tryptophan cultures. The indole continued to increase slowly until a maximum value of approximately 0.5 mM was reached after 7 hours.

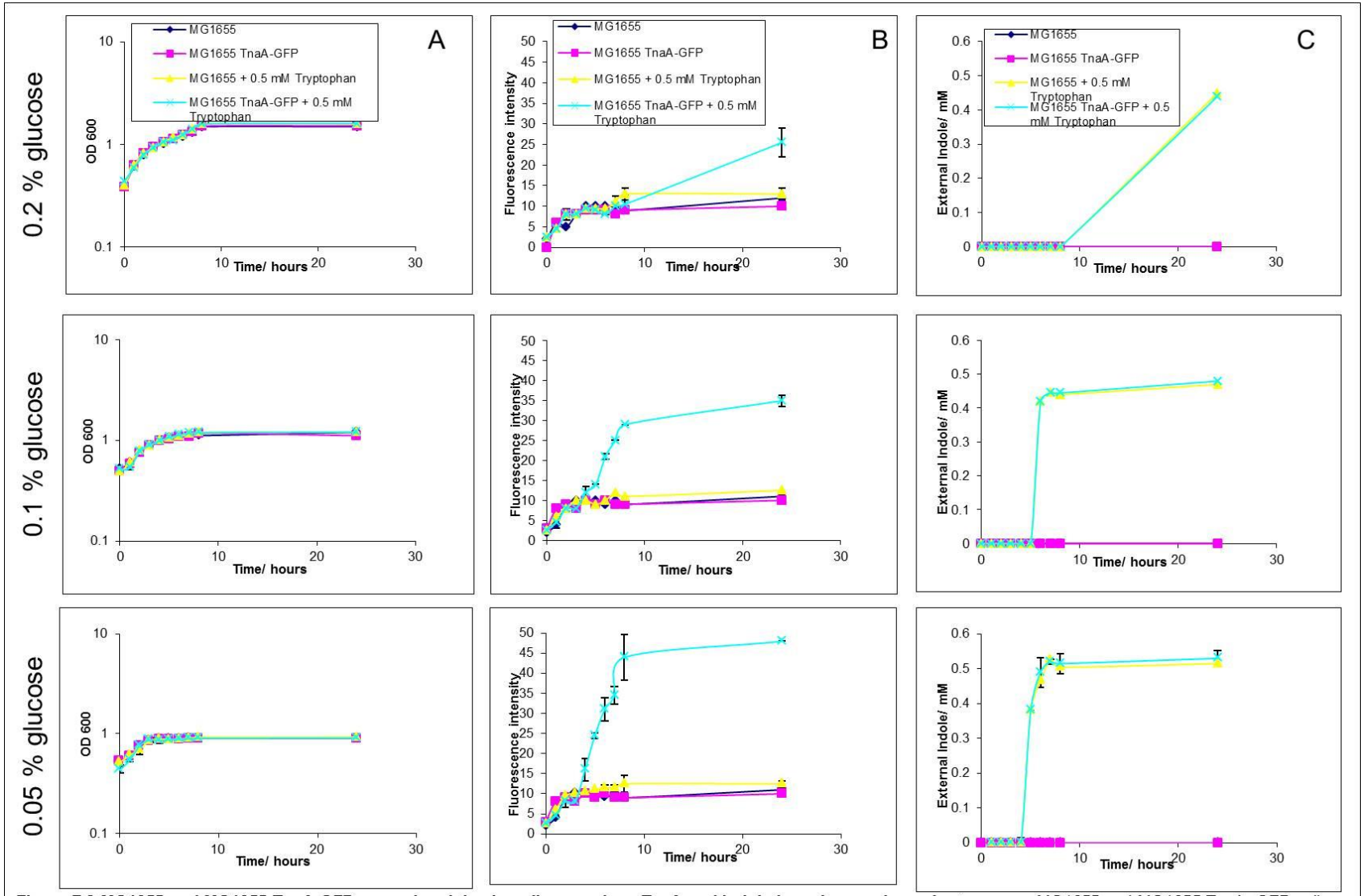


Figure 7.8 MG1655 and MG1655 TnaA-GFP grown in minimal medium produce TnaA and indole in a glucose dependent manner. MG1655 and MG1655 TnaA- GFP cells were grown in minimal medium with 0.2%, 0.1% or 0.05% glucose with and without 0.5 mM tryptophan. Samples were removed hourly for 8 hours then at 24 hours. The OD_{600} (A) and fluorescence intensity (excitation 480, emission 510 nm) (B) was measured . The samples were centrifuged to remove cells. The supernatant was assayed for indole using Kovacs assay (C). Data shown are the mean values ± standard deviation for three independent repeats.

7.9 There is no significant difference in the density or maintenance of cell numbers between wild-type and non indole producing mutant cells when grown in minimal medium.

The results in sections 7.7 and 7.8 showed that, when grown in minimal medium with 0.2% or 0.4% glucose, cells do not express tryptophanase and therefore cannot make indole until after they have entered stationary phase. This contrasts with cells grown in LB medium, which express tryptophanase and make indole during entry into stationary phase (Fig. 3.1). The pulse signalling hypothesis asserts that production of indole at the end of exponential phase regulates proper stationary phase entry and improves long term viability (Fig. 3.3). It therefore follows that if cells grown in minimal medium cannot make indole until they are already in stationary phase, it seems unlikely that indole production would improve long term viability in extended stationary phase.

To test this, overnight cultures of BW25113 and BW25113 Δ*tnaA* were diluted in triplicate into fresh minimal medium + 0.2% glucose with or without 0.5 mM tryptophan to an OD $_{600}$ of 0.05. The cell density (by measuring OD $_{600}$) and cell numbers (by diluting appropriately, plating onto LA plates and counting colonies the following day to obtain CFU X 10^6/ ml) were measured (Fig. 7.9). Samples were taken after 2 days then every 3-4 days subsequently, over a total of 10 days.

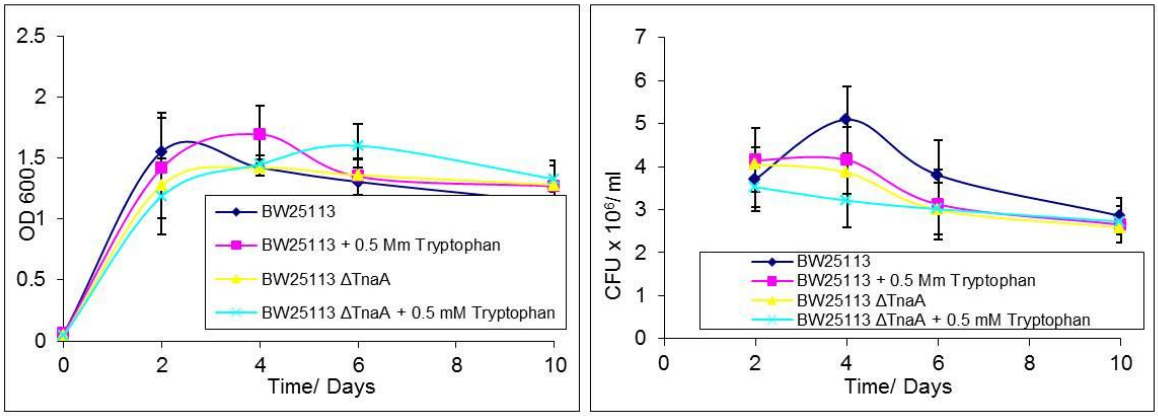


Figure 7.9 The density and cell numbers of BW25113 and BW25113 Δ *tnaA* cells grown in minimal medium with 0.2 % glucose ± 0.5 mM tryptophan are not significantly different. The density (OD $_{600}$: A) and cell numbers (CFU x 10^6/ ml :B) of BW25113 and BW25113 Δ *tnaA* grown in minimal medium with 0.2% glucose and ± 0.5 mM tryptophan added were assessed over 10 days. Data shown are the mean values ± standard deviation for three independent repeats.

The density of all cultures increases from approximately 0.05 to 1.5 after 2 days and the density then stayed approximately constant, over the next 8 days. The number of cells in all cultures was approximately 4 x 10 ^{6}/ ml after 2 days. The cell numbers

of all cultures gradually declined, falling to approximately 3 x 10^{6}/ ml after 10 days. There was no significant difference between the cell numbers in the four cultures.

This is a strikingly different result obtained to when the density and viability of BW25113 and BW25113 Δ*tnaA c*ultures grown in LB medium is compared. Initially, BW25113 cells enter stationary phase at a lower density, but have increased maintenance of cell numbers, across an extended period, compared to BW25113 Δ*tnaA* cells (Fig. 3.2).

It is consistent with the pulse signalling hypothesis that when indole is not produced (BW25113 and BW25113 Δ*tnaA* grown without tryptophan and BW25113 Δ*tnaA c*ultures grown with 0.5 mM tryptophan) there is no difference in the density at which cells enter stationary phase or in the long term cell viability. However, it also shows that when indole is produced once cells have already entered stationary phase (BW25113 grown with 0.5 mM tryptophan), there is also no difference in the density at which cells enter stationary phase or improvement in long term cell viability.

7.10 Discussion

This chapter assessed the roles of tryptophan and glucose in control of the indole pulse. The main conclusions were firstly that the indole pulse requires exogenous tryptophan in the growth medium and secondly that the magnitude of the pulse is dependent on the concentration of tryptophan available. Furthermore, the timing of indole production is glucose dependent and the indole pulse can only confer a viability improvement when the pulse is experience prior to entry into stationary phase. Together these results further emphasise the role of the pulse of indole in determining proper stationary phase entry and subsequent maintenance of cell number. If cells do not experience the pulse, or experience once in stationary phase, rather than on entry into stationary phase, the behaviour of the cells is not changed, and no significant difference between the wild-type and Δ*tnaA* mutant cells is observed.

Previous work had shown that indole production in *E. coli* was determined by the amount of external tryptophan. Tryptophan could be converted into equal amounts of indole, up to concentrations of approximately 5 mM (Li and Young, 2013). It was therefore suggested that the pulse stops simply because all the available tryptophan in the medium was depleted. This chapter provides significant evidence for this

idea, as when additional tryptophan is provided in the growth medium the external indole is increased by equal amounts (Fig. 7.1 and 7.2). Furthermore, when additional tryptophan is provided and the cell associated indole assayed, the pulse is increased in both height and width (Fig. 7.6). This shows that if additional tryptophan is provided, the pulse is extended, strongly suggesting that the observed pulse kinetics observed in LB medium (Fig. 4.1) occur as a result of all available tryptophan being depleted.

The work detailed in this chapter shows that if cells are provided with sufficient amounts of tryptophan, concentrations of indole in the supernatant can reach levels as high as 5 mM. This might therefore suggest that the inhibit of cell division and growth by addition of high concentrations (3-5 mM) of indole to the supernatant (Chant and Summers, 2007, Chimerel *et al*., 2012) is a biologically relevant process, not just on entry into stationary phase, but in response to high tryptophan concentrations too. This would however only be true in a culture which had also run out of glucose as well.

An important question is whether *E. coli* is ever exposed to such high tryptophan levels, to convert into indole, in a natural environment? In the pig digestive tract, the concentration of tryptophan was estimated to be highest in the middle part of the small intestine (~0.5–0.7 mM) and lower in the large intestine (<0.1 mM) (Knarreborg *et al.,* 2002). These levels are comparable to the concentration of tryptophan in LB medium. However, Li and Young (2013) speculate that as tryptophan accounts for up to 0.23% of the weight of animal muscle tissue, this would be equivalent to a concentration of approximately 11 mM, if fully degraded. Whilst acknowledging that this would not be normally available to *E. coli* residing in the gut, they suggest that under some circumstances (e.g. tissue breakdown following severe infection) then *E. coli* may be exposed to significantly higher tryptophan concentrations. They conclude that it is not inconceivable that, in specific environments, *E. coli* may access sufficient tryptophan to produce indole up to 5 mM. However, the key question remains, would the normal environment experienced by cells be more like LB, minimal medium or something else completely different? It is clear that the mammalian gut would provide nutrients, including tryptophan and glucose as described above. The work described above suggests that in order for *E. coli* cells to produce a pulse of indole in such an environment glucose would first have to be depleted and tryptophan be available at

relatively high concentrations. The pulse of indole may potentially provide *E.coli* a competitive advantage over non indole producing species.

Production of indole stopped when a supernatant concentration of approximately 5 mM was reached, even when additional amounts of tryptophan were provided (Fig. 7.2). Li and Young (2013) suggest that one reason for this could be that high concentrations of indole act to inhibit the activity of tryptophanase, by product inhibition. Indeed, tryptophanase from *Bacillus alvei* is inhibited by indole (Hoch and DeMoss, 1973). They also suggest that the activity of the tryptophan transporter TnaB may be inhibited in the presence of high concentrations of indole, as it may require the proton motive force to be intact in order to import tryptophan into the cell (Sarsero *et al*., 1991). As 5 mM indole in the supernatant of a culture would act to dissipate the proton motive force across the membrane (Chimerel *et al*., 2012), no further uptake of tryptophan could occur and so no further indole could be produced.

When cells were grown in minimal medium, in the absence of tryptophan, no indole was detected. This indicates that tryptophanase does not convert significant amount of the internal pool of tryptophan in to indole. Li and Young (2013) suggest a potential reason for this is the relative affinity of tryptophan for tryptophanase compared to other enzymes for which it is a substrate. The measured K_m of TnaA for tryptophan ranges between 0.30-0.60 mM. The K_m of tryptophan-tRNA ligase for tryptophan is 0.017 mM and K_d of the *trp* operon repressor, TrpR, for tryptophan is 0.018 mM. This means that tryptophanase has a much lower affinity for tryptophan compared to other enzymes which use it as a substrate. This can explain why no detectable indole is observed when the cells are grown in the absence of tryptophan.

When cells were grown in minimal medium, in the presence of tryptophan, the timing of indole production was glucose-dependent. It seemed that glucose needed to be depleted, allowing RpoS levels to increase and to trigger tryptophanase expression, in turn allowing indole production. An alternative explanation of this result could be that in the presence of high glucose concentrations indole was not produced due to catboltie repression of the tryptophanase operon. Future work could test this idea, by assessing tryptophanase expression levels in BW25113 *tnaA-GFP* and BW25113 Δ*rpoS tnaA-GFP* cells when grown in minimal medium in the presence of various glucose concentrations.

A result of this glucose effect was a key difference in the timing of indole production when cells were grown LB medium or minimal medium. In LB medium, cells produce indole on entry into stationary phase. When grown in minimal medium, cells produce indole when growth as completely stopped, in stationary phase. As a result of this, the growth and viability of cells grown in minimal media is not affected by the pulse of indole. This causes wild-type and indole non-producing cells to show the same growth phenotypes and long term cell numbers. This further underlines the importance of the timing of the pulse, in helping contribute to the behaviour of cells at the onset of stationary phase.

Chapter 8

Discussion

8.1 Validity of the pulse signalling hypothesis

At the outset of this work, the main aim was to establish if the inhibition of growth and cell division by high concentrations (3-5 mM) of indole were biologically relevant effects. This work has demonstrated that such concentrations do occur naturally, at the onset of stationary phase when indole production is rapidly upregulated. This results in a brief and intense pulse of indole associated with the cells. In the short term, this indole pulse seems to slow growth and cell division on entry into stationary phase, and in the long term improves viability of the culture during starvation (shown in Fig. 8.1). The idea of signalling by a high transient level of indole was coined the pulse signalling hypothesis and important aspects of the hypothesis were tested experimentally.

Results described in chapters 3 and 5 demonstrated that the pulse of cell associated indole was the key regulator for early stationary phase entry, as neither addition of low external amounts of indole or co-culturing Δ*tnaA* mutant cells with wild-type cells was sufficient to restore wild-type behaviour to the Δ*tnaA* mutant cells. In addition to this, work presented in chapter 4 showed that the 60 mM cell associated indole (the peak of the pulse) was equivalent to addition of 4 mM indole to the culture supernatant, which is sufficient to inhibit cell growth and division (Chant and Summers, 2007, Chimerel *et al*., 2012). In chapter 6, the mechanism by which increased indole production is achieved was shown to possibly involve RpoS, which increases tryptophanase gene expression on entry into stationary phase (Fig. 8.1). In addition to this, chapter 7 suggests that the role of the pulse could be to prevent glucose starvation during long term stationary phase, by slowing growth and cell division, ensuring glucose levels are maintained. This helps explain the differences in phenotypes between wild-type and Δ*tnaA* mutant cells, as presented in Fig. 8.1.

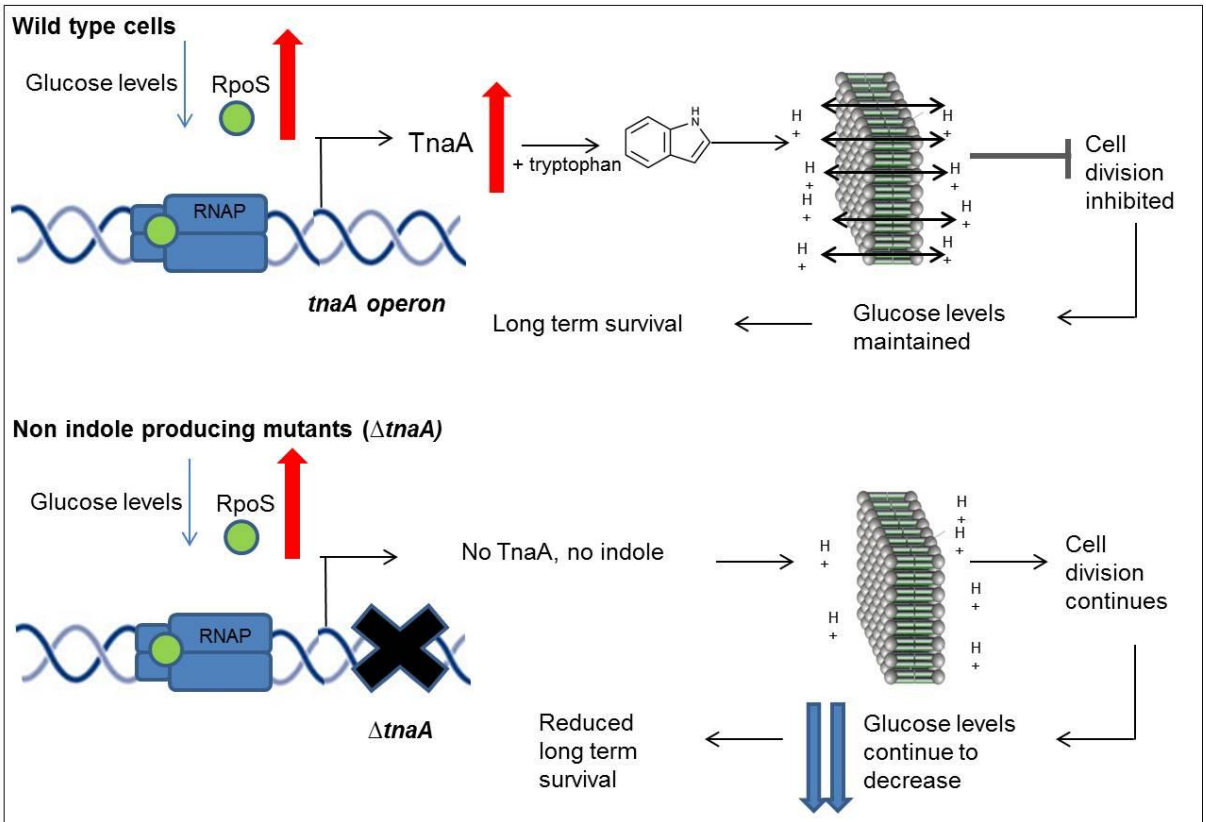


Figure 8.1 The suggested role of glucose limitation in indole production and long term survival. In wild type cells, on entry to stationary phase, glucose levels decrease, increasing RpoS levels, inducing tryptophanase expression. This produces the indole pulse, which dissipates the membrane potential, inhibiting cell division. Glucose levels are maintained , ensuring long term survival. In non indole producing mutant cells on entry to stationary phase, glucose levels decrease, increasing RpoS levels, but tryptophanase expression is not induced and no indole is made. Cells continue to divide, further depleting glucose in the medium, resulting in poor long term survival

8.2 The indole pulse as a novel mode of signalling

Past work on indole signalling has focused on the effects of low (0.5- 1 mM), persistent levels of indole (Lee and Lee, 2010). Whilst there is no doubting the effect of indole at these levels to many biological processes, this work has demonstrated a novel type of indole signalling, whereby high, transient levels of indole can have important roles in *E. coli* physiology. The pulse signalling hypothesis expands the biological roles of indole to include growth and cell division inhibition.

Previously, indole has been referred to as a quorum sensing molecule (Lee *et al*., 2009, Walters and Sperandio, 2006). Quorum sensing, a term first used by Fuqua *et al.* (1994) can be described as a type of cell-to-cell communication, in which the expression of targets genes is modulated in a cell density dependent way. Induction of gene expression requires a critical concentration of a diffusible signal molecules or autoinducers, in the local environment.

In a recent review by Lee and Lee (2010), they suggested that indole fulfilled the 4 key criteria described by Winzer *et al.* (2002) in order to be termed a 'cell to cell signal molecule' (CCSM). Firstly, the CCSM must be made at a specific stage. Secondly, the CCSM accumulates externally and is recognised by a specific receptor. The third condition is that accumulation of the CCSM generates a concerted response. The final condition is that the response by the cells extends beyond physiological changes to remove or detoxify the molecule. Winzer *et al.* (2002) also state that if a molecule is a CCSM then it may also be a *bona fide* quorum sensing molecule.

Low, persistent indole signalling seems to fit at least some of these criteria. Indole is made on entry into stationary phase and accumulates externally as shown in previous work (Chant and Summers, 2007) and in Chapter 3. Furthermore, using conditioned medium, or chemical complementation, in previous work has shown to be effective in mediating effects of low persistent levels of indole, such as induction of gene expression (Hirakawa *et al.,* 2005). However, this thesis suggests that in stationary phase entry, external accumulation of indole is irrelevant, and that it is the cell associated pulse of indole which is crucial here. Addition of 1 mM exogenous indole to mutant cells or co-culture of mutant cells with wild-type indole producing cells was insufficient recreate the wild-type phenotype regarding entry into stationary phase or long term viability. This suggests that the indole pulse signal does not qualify as a CCSM or quorum sensing molecule.

Furthermore, indole also appears to fail the condition that the CCSM is recognised by a specific receptor. Lee and Lee (2010) concede that no receptor for indole is currently known. Indeed work by Chimerel *et al*. (2012) and this thesis suggests that during entry to stationary phase the target of the indole pulse is likely to be the cell membrane itself.

However, some criteria are met by indole. Both other work and this thesis agree that indole accumulation does generate a concerted response. The response by the cells to indole also extends beyond physiological changes to remove or detoxify the molecule, which both other work and this thesis agree with also.

There may even be some doubt that low, persistent levels of indole detected in the supernatant once the cells are in stationary phase act in a way that is analogous to a quorum sensing molecule. Winzer *et al.* (2002) highlight a previous report of

indole inducing gene expression, where chemical complementation with indole induced the genes to a considerably smaller extent (Wang *et al*., 2001). Indeed this is not the only example where chemical complementation is insufficient to restore the effect of endogenously produced indole. Enteropathogenic *E. coli* required the tryptophanase gene in order to be virulent in a *C. elegans* model. Complementation of non indole producing mutant enteropathogenic *E. coli* with small amounts of external indole was shown to be ineffective in restoring pathogenicity (Anyanful *et al*., 2005).

In mathematical models of quorum sensing it is typically assumed that the autoinducer is always made and secreted at a constant rate (Nadell *et al.,* 2008). This thesis, along with previously published work (Chant and Summers, 2007), provides evidence that indole production and secretion in *E. coli* does not occur at a constant rate. Recently however, it has been recognised that production and diffusion rates of CCSMs are likely to play a key role in signalling (Boyer and Wisniewski-Dye, 2009). There is clearly uncertainty in the literature regarding the role of indole as a CCSM and quorum sensing molecule, with Winzer *et al.* (2002) suggesting that indole would not qualify as a CCSM, whilst Lee and Lee (2010) and others claim indole is a quorum sensing molecule.

This thesis proposes a new type of indole signalling, whereby increased indole production rates result in high transient levels of indole which influence cell division and growth *via* effects on the cell membrane. This signalling is completely distinct from quorum sensing type signalling as described above, mainly because its effects cannot be induced by conditioned medium or chemical complementation and the indole is not made at a constant rate. It is likely that some roles on indole such as mediating changes in gene expression are mediated by low persistent concentrations, but that others absolutely require high transient concentrations of indole. This suggests that there are two distinct modes of indole signalling, one which occurs at high, transient levels of indole and one which occur due to low, persistent levels of indole. It is interesting to speculate that the confusion and contradictions within the indole signalling literature may, in part, be due to the fact that the effects of high, transient levels of indole have not been previously appreciated.

Another factor which distinguishes indole signalling from other bacterial signals such as nucleotide messengers is that in most cases (e.g. cAMP, di-GMP) there are

two enzymes controlling the cellular levels of the signal molecule, one which produces the molecule and another to hydrolyse the molecule. However, no enzyme exists to hydrolyse indole. Indole is made, it diffuses across the cell membrane into the culture supernatant where it remains. It is interesting to speculate that this could be the way cells deal with indole once it has been produced as the pulse.

8.3 Remaining questions and future work

The aim of this work was to investigate whether inhibition of cell division and growth by high levels of indole were biologically relevant processes. This work has demonstrated that at the onset of stationary phase, indole production is upregulated rapidly resulting in a pulse of indole sufficient to inhibit cell division and growth. However, some important questions still remain and this section will suggest future work which could help answer these questions.

8.3.1 Would an artificial indole pulse in mutant cultures restore the wild-type phenotype?

It is highly likely that the pulse of indole contributes to proper stationary phase entry in wild-type cultures as mutant cultures could not have this behaviour restored by addition of low external amounts of indole or by co-culturing with wild-type cells. It would be expected that if the pulse of indole could be artificially reproduced in the mutant cultures then the stationary phase phenotype should be restored. Future work will focus on trying to provide the Δ*tnaA* mutant cells with an indole pulse of similar intensity and duration to that observed in wild-type cells. Initial attempts to do this have encountered problems. It is easy to provide a large amount of indole, by simply adding it to the culture medium. However, it is significantly more difficult to remove the indole from the cells; early attempts did this by centrifugation of the cells, followed by resuspension in spent medium. Alternative future approaches could try to recreate the pulse in mutant cells by having the tryptophanase gene present on an inducible plasmid, allowing production of endogenous indole when required.

8.3.2 Does the pulse directly depolarise the cell membrane and inhibit cell division?

Another key question to address is whether the primary effect of the indole pulse is to depolarise the cytoplasmic membrane, and hence inhibit cell division. Previous work showed that the addition of between 3-5 mM indole to the culture supernatant depolarises the cytoplasmic membrane and arrests growth and division (Chant and Summers, 2007, Chimerel *et al.,* 2012). The pulse signalling hypothesis predicts that membrane depolarisation should be seen during stationary phase entry. It may be difficult to detect short-term membrane depolarisation but work in collaboration with the Keyser group (Cavendish Laboratory) could explore several possible approaches. For example, use of the proteorhodopsin optical proton sensor (PROPS) has been shown to be effective in detecting rapid voltage changes (Kralj *et al*., 2012). A non-invasive technique could also be developed for monitoring membrane potential such as the analysis of low frequency dielectric dispersion properties of an *E. coli* suspension that has already provided evidence for changes in membrane polarisation during different *E. coli* growth phases (Bot and Prodan, 2010).

8.3.3 What other effects could the indole pulse have?

It is interesting to speculate what other roles the pulse of indole may have in addition to the inhibition of cell division *via* an ionophore base mechanism (Chimerel *et al*., 2012). The partition coefficient of indole shows that indole has an approximately 90-fold greater affinity for the cell membrane, than either the aqueous culture medium or the cytoplasm. Therefore by far the highest concentrations of indole during the pulse will be in the cell membrane. Such large quantities of indole inside the membrane are likely to affect the physical properties such as the charge distribution, intrinsic structure and fluidity. This, in turn, is likely to affect the function of proteins within the membrane. One previously reported example of a membrane proteins affected by the presence of indole are MinCD and FtsZ (Chimerel *et al*., 2012). In this case the effect is mediated by indole acting to dissipate the proton motive force. In theory, any membrane protein which needs an intact proton gradient to localise and/or be active will be affected by the presence of indole in this indirect manner. However, it could also be imagined that proteins in the membrane could be affected directly by the presence of large amounts of indole.

8.3.4 Is the pulse produced in response to other stresses?

Investigations into the regulation of the pulse show that an RpoS directed increase in tryptophanase expression may be responsible for increased indole production. In the case of stationary phase entry, nutrient limitation is likely to be the main cause of increased RpoS levels. However, RpoS accumulation is not limited to stationary phase entry, it is also observed in response to a variety of stresses including altered pH and osmotic or temperature shocks (for review see Battesti *et al.,* 2011). This raises the possibility that an indole pulse may be an integral part of a general cellular response to stress. Future work could test whether an indole non-producing mutant shows lower viability than the wild-type in cultures exposed to these various stressed. This will indicate whether indole production has any role in stress protection. It will be important to distinguish between protection by a persistent low concentration of indole and a transient high concentration during the pulse. An attempt will be made to restore the viability of the stressed cells by addition of low concentration of indole (1 mM) to the mutant culture. If this is unsuccessful (as demonstrated for the long term survival in stationary phase culture; Fig. 3.2) it will be evidence of a role for pulse signalling in the stress response. In these cases, *tnaA-GFP* strains can be used to monitor tryptophanase expression and cell-associated indole during and after the imposition of stress for evidence of a transient indole pulse in the cells.

8.3.5 How do high concentrations of indole inhibit growth?

This work has demonstrated the importance of inhibition of growth and cell division, by indole. The mechanism by which indole inhibits cell division is well understood, indole acts as a ionophore to dissipate the membrane potential, preventing proper oscillation of the Min proteins, in turn inhibiting FtsZ localization (Chimerel *et al*., 2012). However, the mechanism by which high levels of indole inhibit growth in *E. coli* is not fully understood. Addition of indole to *E. coli* has been shown to cause a slow depletion in the ATP levels in the cells (unpublished, Chris Field) which may be related to its ability to halt growth. Furthermore, in *Pseudomonas putida* indole increases the $NADH/NAD^+$ ratio (Kim *et al*., 2013), which may contribute to slower growth. Future work could identify if the $NADH/NAD^+$ ratio in *E. coli* is perturbed by the presence of indole, to see if this contributes to inhibition of growth.

8.3.6 Is the indole pulse relevant to *E. coli* growing in natural environments?

This work considers indole production in bulk broth culture, so it is important to note that the pulse duration reported in Chapter 3, approximately 30 minutes, is an average over all cells in the culture. If there is significant population heterogeneity in the time of pulsing, the pulse duration in individual cells may be shorter and the maximum concentration higher than the values presented here. Additionally, in broth culture secreted indole is rapidly diluted by mixing throughout the culture, which has a significantly larger volume compared to a single cell. This seems to be poor model for natural environments such as the human gut, or where bacteria grow on a solid substrate as colonies or in biofilms. It is tempting to suggest that in these native environments secreted indole may remain longer in the vicinity of the producer cells, resulting in higher local concentrations and potentially increasing the effectiveness of pulse signalling. Future work could therefore extend the comparison of wild-type and indole non-producing strains to cells growing within a three-dimensional matrix of nutrient soft agar (Wimpenny, 1995). Modified assays for growth, viability and indole production could be developed for use in this semi-solid environment. This approach could also facilitate single cell studies will help address the issue of heterogeneity.

Another interesting question relates to indole production in the native environment of *E. coli*. Would it be advantageous in the human gut to produce a pulse of indole to assist long term survival during starvation? *E. coli* tend to be thought of as living a feast and famine lifestyle so intuitively it seems it might. However it could also be imagined that it might be advantageous to not make indole, essentially to 'cheat' and have an initial growth advantage followed by using up the residual resources set aside by wild-type counterparts. It would be interesting to see if tryptophanase mutants occur in natural populations of *E. coli* and if so, at what levels. There is already evidence that RpoS mutants occur at significant levels in natural populations (Synder *et al*., 2012) and an RpoS mutant is likely to have altered tryptophanase expression, and hence altered indole production.

8.4 Conclusions

In conclusion, this thesis has identified a new mode of indole signalling in *E. coli,* whereby a pulse of indole regulates growth and cell division on entry into stationary phase. It remains interesting to see if this type of signalling is of widespread

significance in the native environment of *E. coli* and in response to other stresses and whether it potentially applies to other signalling molecules.

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