LATE STATIONARY PHASE-SPECIFIC GENE EXPRESSION IN *E. COLI*

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

MASTER OF SCIENCE (2016)

McMaster University

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TITLE: Late stationary phase-specific gene expression in *Escherichia coli*

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NUMBER OF PAGES: x, 135

ABSTRACT

Interest in stationary phase and adaptation mechanisms to non-optimal conditions led to the discovery of RpoS as a growth phase-dependent sigma factor. Subsequently, studies aimed at profiling members of the RpoS regulon have provided insights into the stationary phase of growth. At the same time, stationary phase physiology, which now includes formation of biofilms and persistence, is characterized by many RpoS-independent but growth phase-dependent genes, which have received less attention. Obtaining a comprehensive understanding of stationary phase requires a comprehensive profiling of these genes. We employed a plasmid-based promoter *gfp::mut2* fusion library of 1920 promoters to screen for promoters active in planktonic culture during long-term incubation. Two hundred and twenty nine identified promoters fall into molecular function categories of catalytic activity, transport, stress-response, replication inhibition, and transcription. Only 35 of these are positively-regulated by RpoS and 15 are negatively-dependent during early stationary phase. We found that levels of *rpoS* transcript peak during fast growth conditions of early stationary phase (OD600 = 1.5) and decrease thereafter. Induction of tryptophanase, which produces indole, was discovered to be RpoS-independent but stationary phase-specific at the transcript and protein levels. Furthermore, RpoS serves to limit TnaA levels following initial induction in a dose dependent manner. At the transcript level, RpoS positively regulates TnaA in exponential phase (OD600 = 0.3) but negatively in early stationary phase (OD600 = 1.5). The mechanism underlying this switch is unknown. Thus, mature stationary phase cultures are characterized by a physiology that is at least partially, if not completely, distinct from

early stationary phase cells and RpoS-independent but stationary phase-specific genes play an important role during prolonged starvation.

ACKNOWLEDGEMENTS

First and foremost, I want to thank Dr. Schellhorn for accepting me as a graduate student and providing me all the necessary resources and guidance in this project. I whole-heartedly want to thank him for providing me the support and intellectual freedom to explore my scientific interests and at the same time steering me in the right direction and improving my skills in terms of writing and experiments. Thank you once again!

I whole-heartedly want to thank Dr. Elliot, my co-supervisor, for her guidance and support. Her advice and input into my project and my writing have been invaluable in improving my skills.

I want to thank all members of the Schellhorn lab (both past and present) for making the past two years, a memorable time. I have had wonderful time with Sakis, Mahi, Vivian, Cathy, Ashish, Rachel, Rachelle, Steve, Shirley, Paul, James, Srikar, Kristine, and Bansri. Going for coffee with Mahi and Sakis was wonderful. I will particularly miss conversations and spending time with Sakis and want to thank him from the bottom of my heart for his support, insight, and for being a great friend. Finally, I thank my family for their unconditional support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1: Stationary phase-specific gene expression in *E. coli*

1.0 Introduction

As a model organism, *Escherichia coli* has been critical in providing insights into genetic and regulatory mechanisms shared by all organisms as well as the role of gut microbiota in human development and disease (1, 2). The life of a single *E. coli* cell can be divided into its primary and secondary habitats (3, 4). The primary habitat consists of nutrient rich and anaerobic conditions found within the colon. This environment supports stable growth due to constant influx of nutrients into the gut. The human intestine upon birth is sterile and the process of colonization by *E. coli* and other gut microorganisms begins soon after birth and is influenced by factors like mode of delivery (1), early life nutrition (5), and follows a temporal order of colonization by different species (6). Upon excretion outside the host, the bacterium encounters its secondary habitat. In secondary habitats, *E. coli* is subjected to more unfavourable variation in the physical environment, which may consist of soil, water, or sediment (3). Despite the non-optimal conditions found in the primary as well as secondary habitats, *E. coli* (at least at the population level) has a remarkable ability to survive until conditions optimal for growth occur (7, 8). There have been many insights into the gene regulatory mechanisms responsible for adaptation to non-optimal conditions. The stationary phase (9-13), biofilms (14-19), and persistence (20-22) are distinct physiological growth states when *E. coli* expresses many stress tolerance and survival genes compared to fast-growing exponential phase cells. Besides the differences in transcriptome and the proteome between these distinct modes of life, there are morphological differences. For example, exponential phase cells are flagellated and motile while cells in stationary phase lack flagella (23). Ever since the recognition of

stationary phase as a distinct physiological state from exponential phase, understanding of regulatory interactions governing this phase of bacterial life has greatly improved. The following is a discussion of major processes occurring in stationary phase planktonic cells.

1.1 Response to non-optimal growth conditions

1.1.1 Specific and the general stress response

In the environment outside the host organism, bacteria rarely encounter conditions that support fast growth and are frequently faced with a challenge that poses a physical damage threat or are starved for a particular nutrient required to sustain growth and metabolism (4). It is not surprising then that *E. coli* and other bacteria have dedicated regulatory networks that mediate the cellular response to such conditions (24). Many of these responses are specific for a particular stress condition and are aimed at either removing the stressor or adopting a physiology that limits damage until the stressor is removed (25). For example, upon a shift of temperature from 30 °C to 42 °C, the heat shock response is induced under the control of heat shock sigma factor, RpoH (26). For 5-10 minutes after temperature upshift, transcription and translation of *rpoH* increases leading to higher intracellular RpoH levels (27), which upregulates genes whose products are responsible for stabilizing and re-folding denatured proteins, genes whose products are responsible for proteolysis of misfolded proteins, and changing the composition of the plasma membrane (28, 29). A model for explaining the induction of RpoH is that RpoH synthesis is inhibited by molecular chaperones DnaK, DnaJ, GrpE. Titration of these chaperones by denatured proteins during heat shock relieves this inhibition (30). Another

example of a response to specific stress is the glutamate-dependent acid stress response (31, 32) mediated by the proteins encoded by the genes of the acid fitness island (which include the glutamate decarboxylases GadA and GadB that remove an intracellular proton in a decarboxylation reaction of glutamate). In contrast to response to a specific stress such as the acid resistance response or the heat shock response, the stationary phase sigma factor RpoS is induced in response to multiple non-optimal conditions and controls about 10% of the *E. coli* genome during entry into stationary phase (11). These inducing conditions include osmotic shock (33, 34), oxidative stress (35-37), heat stress (38), and entry into stationary phase (11, 13, 24). This suggests the idea that a particular condition such as carbon starvation may be a possible predictor for another stress and it may be advantageous to up-regulate the expression of multiple protection functions under the control of a central regulator such as RpoS (39). For example, heat shock induces RpoS through upregulation by DnaK (40). It is interesting to note however, that the set of genes controlled by RpoS depends on the specific inducing signal. The genes induced during transition to stationary phase are different from genes induced during heat shock (40). Similarly acid stress on exponentially growing bacteria induces a different set of genes under the control of RpoS than those induced during transition to stationary phase (41). An explanation for this is that many of the genes that are differentially induced under distinct stress conditions require additional regulators to modulate RpoS binding to the promoter or alter its activity (39).

1.1.2 RpoS-mediated stationary phase adaptation

RpoS was recognized in the early 1990s as a growth phase-dependent regulator responsible for induction of stress-induced genes and genes induced during transition to stationary phase (24, 42). The importance of RpoS as a regulator for survival in non-optimal conditions is corroborated using *rpoS* mutants that have reduced viability upon exposure to non-optimal growth conditions (9, 43). Due to interest in elucidating the mechanisms of survival in stationary phase, a relatively large number of studies have examined the extent of and the regulatory pathways within the RpoS regulon using transcriptional reporters or microarrays (9-11, 13). In addition to validating the regulation of previously known-prototypical RpoS regulated genes including *katE, dps, osmY,* and *otsA* microarray analysis was used to discover that a large set of genes is under negative control by RpoS. Upon *rpoS* deletion during early stationary phase (OD600 = 1.5), 252 genes are upregulated, while 180 genes are downregulated. A chromosomal region containing rac prophage genes, the flagellar genes (fifty genes controlling motility), and some enzymes of the TCA (tricarboxylic acid) cycle are negatively regulated by RpoS (11). In addition, tryptophanase, which catalyzes a reversible conversion of tryptophan to indole, pyruvate and ammonia, is negatively regulated during early stationary phase (11). In a similar study, RpoS induced the expression of genes required for amino acid metabolism, protein synthesis, iron utilization, and the tryptophanase enzyme. In addition to modulation of tryptophan levels in stationary phase (through conversion of tryptophan to indole by tryptophanase), RpoS controls intracellular levels of arginine (through *artP* and *artI* transporters) and asparagine (through *ansP* transporter). In addition to limiting

oxidative damage through catalases (*sodC* and *katE*), RpoS induces bacterioferritins like the small DNA binding protein Dps, which bind and chelate iron, preventing oxidative damage (10).

In another study, WT and *rpoS*::Tn10 (a transposon insertion mutant in the *rpoS* gene) mutants were compared under three conditions using microarray analysis: transition to stationary phase in LB, 20 minutes after addition of NaCl (0.3 M) in minimal medium for osmotic upshift, and 40 minutes after acidification of rich medium by MES (4-morpholine-methanesulfonic acid) (41). In total, 481 genes were positively regulated by RpoS and 95 were negatively regulated in these conditions. Out of these, only 140 genes are consistently positively regulated under the three conditions and these comprise the core set of genes under the control of RpoS. An interesting regulation aspect revealed by this study was the modular design of some RpoS-dependent genes. Genes within the RpoS regulon can be controlled directly by other environmental factors. Expression of glutamate-dependent decarboxylase*s gadA* and *gadB* is RpoS-dependent during entry into stationary phase but not during acid stress imposed on exponentially growing bacteria (41).

The overall set of genes controlled by RpoS depends on the growth conditions. This idea is supported by the observation that expression and composition of the RpoS regulon in minimal medium is different from that during growth in rich medium in both exponential phase and during entry into stationary phase (44). This might be due to levels of RpoS itself since RpoS is constitutively expressed in minimal medium or due to additional regulatory proteins interacting with RpoS. Supporting the later is the

observation that RpoS-controlled genes in exponential phase in rich medium are different than those of the stationary phase and Crl serves as an activator of RpoS-dependent promoters in exponential phase (45). Furthermore, composition of the RpoS regulon varies among strains of *E. coli* and can include genes needed for survival of strains adapted to specific niches and lifestyles such as pathogenic strains. In *E. coli* O157:H7, *rpoS* deletion affects the expression of about 1000 genes in early stationary phase and only 11 genes in exponential phase in contrast to K12 MG1655 laboratory strain, which has more genes under the control of RpoS in exponential phase (9). *tnaB*, encoding a tryptophan transporter, and *tnaA* (tryptophanase), are expressed lower in the *rpoS* mutant in stationary phase. This is in contrast to the *E. coli* K12 MG1655 in which *tnaA* is under negative control by RpoS in early stationary phase (11). Additionally *cstA* is positively regulated in contrast to negative regulation in K12 MG1655. The TCA cycle genes and the motility genes are negatively regulated by RpoS in K12 MG1655 but no differential expression occurs in O157:H7 (9, 11). This suggests that negative regulation by RpoS is also strain specific. Eighty percent of the RpoS-controlled genes in O157:H7 are strain-specific suggesting that the composition of the RpoS regulon is highly divergent (9, 11).

1.2 Other regulators in stationary phase

1.2.1 Role of Lrp, cAMP, and H-NS

The differences in gene regulation and regulon composition depending on growth conditions can be due to additional regulators acting together with RpoS at promoters that closely resemble promoters of the housekeeping sigma factor RpoD (25, 46). Increasing evidence has suggested a role for additional regulators in the modulation of genes

controlled by RpoS. An example of this is the *csiD* (*c*arbon *s*tarvation *i*nduced) gene. Expression of *csiD* occurs from a single promoter, which is preferentially dependent on RpoS for transcription initiation and is also activated by cAMP-CRP (cAMP receptor protein). Furthermore, in the presence of leucine in the growth medium, the promoter is slightly upregulated indicating positive regulation by the leucine responsive Lrp transcription factor. Under nutrient limiting conditions, induction of *csiD* is observed specifically during carbon limitation. Induction is not observed in exponential phase cells exposed to high salt concentration, cells exposed to heat-shock, or during nitrogen or phosphorus downshifts (47) although all these conditions lead to accumulation of RpoS. Interestingly, Lrp (responsible for promoting anabolic functions during nutritional downshifts) is induced during entry into stationary phase in rich medium and is positively controlled by the universal stress alarmone ppGpp (48). RpoS also interacts with other regulators in controlling the *osmY* and *csgBA* promoters. RpoS is a positive regulator of *osmY* expression. Exogenous expression of RpoS from a multi-copy plasmid results in a small induction of *osmY* suggesting that other regulators participate in controlling the *osmY* promoter. During growth in both rich and minimal media, the *osmY* gene is under negative control of Lrp during transition to stationary phase. In minimal medium, growth phase-dependent induction of *osmY* in an *lrp rpoS* double mutant occurs due to loss of Lrp-mediated repression during this transition. Furthermore, osmotic regulation of *osmY* occurs independently of Lrp and RpoS when cells are challenged with 0.3 M NaCl. In addition, cAMP-CRP represses the *osmY* promoter during entry into stationary phase and this repression is independent of RpoS. In rich and glucose minimal media, a mutation in

the *himA* gene, which forms one of the subunits of the IHF (integration host factor) dimer, leads to a premature and a higher final stationary phase expression of *osmY*. This effect is also RpoS-independent since repression of *osmY* by HimA is also observed in *rpoS* mutant background (49). *E. coli* attaches to fibronectin using curli organelles, which are positively regulated by RpoS during stationary phase transition. Curli are encoded by the di-cistronic *csgBA* operon. In the absence of the nucleoid associated protein H-NS, expression of *csgBA* occurs using the housekeeping sigma factor RpoD in a growth phase-dependent manner. This suggests that the chromosomal structure, which is a function of this type of nucleoid associated protein, plays a major role in determining sigma factor specificity and thereby controlling gene expression (50). The role of H-NS in modulating RpoS-dependent genes is further supported by the observation that RpoS levels are higher in an *hns* background and that this is concomitant with upregulation of certain RpoS-dependent genes (51). Upon *hns* inactivation, 22 RpoS-dependent proteins show elevated levels including RpoS itself. For example, in both complex and glucose minimal media, inactivation of *hns* causes upregulation of *osmY::lacZ* transcriptional fusion during exponential phase. Not all *rpoS*-dependent genes are subject to repression by H-NS however (52).

1.2.2 Nucleotide messenger: ppGpp

The nucleotide messenger guanosine 5′, 3′ bispyrophosphate (ppGpp) elicits the stringent response to amino acid starvation by affecting transcription in a global manner (53-55). ppGpp is synthesized by a ribosome associated protein, RelA, which senses amino acid starvation through contacts with deacylated tRNA at the A site of the

ribosome (56). RelA converts GDP (guanosine diphosphate) to ppGpp and GTP (guanosine triphosphate) to pppGpp in a phosphorylation reaction where ATP donates a phosphate group (57). SpoT is a cytosolic enzyme (58) that synthesizes ppGpp under starvation conditions other than amino acid starvation and can also function as a hydrolase using ppGpp as a substrate (54). ppGpp accumulation during amino acid starvation like that experienced during entry into stationary phase suppresses transcription from ribosomal RNA promoters and shifts the physiology of the cell from that of fast growth to a more dormant and stress tolerant state with resources directed towards amino acid biosynthesis (54, 59-62). $ppGpp^0$ strain (*relA spoT* double mutant) is therefore auxotrophic for 11 amino acids (63, 64). Genes under the control of ppGpp under distinct stress conditions have been studied. One study examined the components of the stringent response induced after serine hydroxymate (SHX) treatment of cells growing in MOPS (3-(*N*-morpholino)-propanesulfonic acid) medium supplemented with all 20 amino acids and glucose by comparing the WT and *relA* mutants. The WT strain displayed differential expression of about 500 genes in a temporal manner while the *relA* mutant showed a delayed response (termed the "relaxed" response). One hundred and ten genes were differentially regulated upon SHX treatment within the first five minutes of treatment. Promoter activity from the stable RNA promoter of *rrnB* operon, measured by quantifying the leader region transcript, declined in the K12 MG1655 while only a modest reduction was observed in the *relA* mutant. Thirty-seven tRNA genes, including the genes within ribosomal RNA operons, were downregulated by ppGpp. Seven out of eight amino acid biosynthetic operons that are controlled by an upstream transcriptional

attenuator were downregulated however, the transcription from the promoter was upregulated. This may be due to the presence of all 20 amino acids in the growth medium. Genes responsible for motility were downregulated. The alternative sigma factors RpoH, RpoE, RpoS, the anti-sigma factor Rsd, universal stress proteins, cold shock proteins (CspA, CspG, CspB) were induced. CspD is a DNA replication inhibitor that is also under positive control of ppGpp. Toxin anti-toxin modules *relBE* and *chpBI-chpBK*, ribosome modulation factor (*rmf*) were positively controlled by ppGpp, (65) consistent with previous reports (66).

Another study used limiting amounts of isoleucine to induce amino acid starvation response in the presence of all other 20 amino acids in MOPS medium with glucose to study global gene expression in log-phase and following growth arrest in the WT and $ppGpp^0$ strains. One thousand and twenty-four genes were differentially expressed under starvation conditions and included the genes belonging to the general stress response, metabolism, and uncharacterized genes. Compared to the WT, the $ppGpp^0$ strain had a diminished ability to downregulate translation apparatus genes and upregulate RpoS-dependent stress response genes. Upon entering starvation, the WT strain altered central metabolism such that all pathways including glycolysis, TCA cycle, and pentose phosphate pathway were activated so as to produce the amino acid available in limiting amounts (isoleucine in this case). For example, genes coding for the enzymes for branched amino acid synthesis were upregulated. Many of the metabolic genes of central metabolism were not induced in the $ppGpp^0$ mutant. Furthermore, metabolic ability of the $ppGpp^0$ strain to use non-preferred carbon sources declined as assessed using Biolog

assays (63).

Crosstalk between ppGpp and RpoS was previously known and it was proposed that ppGpp acts as an inducer of RpoS during stationary phase (67). The activity of the RpoS-controlled acid phosphatase (AppA) is elevated during transition from exponential to stationary phase only in the WT strain but not in $ppGpp^0$ strain cultures grown in rich medium. After overnight incubation however, both strains had a similar level of AppA activity. During transition from exponential to stationary phase in rich medium, both post-transcriptional and translational regulation play a positive role in increasing cellular levels of RpoS. During growth in LB, the $ppGpp^0$ mutant accumulated RpoS at a lower level. After overnight growth however, RpoS reached similar levels to the WT (and hence the same expression of AppR). In MOPS minimal medium, depletion of glucose or phosphate leads to RpoS accumulation. In the $ppGpp^0$ strain, this accumulation was slightly reduced. In a strain with higher than normal levels of $ppGpp^0$ due to exogenous expression of the catalytic subunit of *relA*, the levels of RpoS were also elevated (67). It is worth noting however that $ppGpp^0$ affects transcriptional elongation rather than initiation at the *rpoS* promoter primarily responsible for stationary phase induction (68). The effect of ppGpp on RpoS-dependent stress response genes are not solely due to upregulation of RpoS as ppGpp can directly stimulate RpoS-dependent promoters (69). Other studies conclude that ppGpp preferentially promotes binding of alternative sigma factors to the core RNAP (RNA polymerase), thereby altering sigma factor competition and gene expression (70). The effects of ppGpp on stress response genes are not just limited to RpoS-dependent genes. ppGpp also activates promoters of genes not controlled by RpoS during starvation.

The *uspA* gene encodes a universal stress protein induced during starvation. ppGpp can override repression of the promoter of *uspA* by FadR and also directly stimulate the promoter during inducing conditions (71, 72).

1.2.3 Interspecies signalling molecule: Indole

Indole is an important signalling compound produced by a large number of Gram-positive and Gram-negative organisms, in response to environmental signals (73, 74). *E. coli* can produce large amounts of indole by conversion of extracellular tryptophan to indole, pyruvate, and ammonia, which is a reversible reaction catalyzed by the enzyme tryptophanase (encoded by *tnaA*). During growth in rich media, *E. coli* produces indole primarily during entry into stationary phase with extracellular indole concentration typically reaching 0.8 mM (75). The final concentration reached in a culture is dependent quantitatively on the initial exogenous tryptophan available in the growth medium with the upper limit concentrations being 5.0 mM (76). Although the pathway of indole production by *E. coli* and related bacteria is well characterized, the extensive roles of indole in diverse cellular processes have only recently emerged. When five mM exogenous indole is added to exponentially-growing cultures, cell division is reversibly inhibited due to indole-mediated dissipation of the proton gradient across the cell membrane. This is because indole acts as an ionophore preventing the formation of the FtsZ ring required for cell division by reducing the promotion motive force (PMF) across the cell membrane (77). Multicopy plasmids can recombine with themselves, which can interfere with stable inheritance of plasmids. Inhibition of cell division by indole can occur during plasmid recombination, leading to inhibition of cell division until plasmids

are resolved (78). During transition to stationary phase, intracellular indole levels rise rapidly resulting in a transient high concentration (60 mM). This pulse of indole appears to promotes long-term cell viability in WT cells compared to indole non-producing mutants by halting growth prior to exhaustion of nutrients (75). In addition to its effect on replication, indole has key roles in drug tolerance (79), acid resistance (80), biofilm formation (15, 16, 81, 82), and persistence (83). Persisters are cells with low enough metabolism to tolerate antibiotic stress and reach about 1% of the total cell number in stationary phase cultures. Expression of the toxin-encoding gene *yafQ* from the *yafQ-dinJ* dicistronic operon reduces indole signaling by reducing both TnaA and RpoS levels, which leads to increased persistence (83). Further supporting its role in stress tolerance, indole induces the expression of some multidrug exporter genes encoded in the *E. coli* genome in a dose-dependent manner (79). Additionally, indole increases acid resistance through increased expression of glutamate decarboxylase acid resistance system. When exponentially-growing cells are treated with exogenous indole prior to acid stress, survival is enhanced (80) through increased expression of acid resistance genes including the *gadABC* cluster. Role of indole signalling in biofilm formation is complex with indole proposed to be either a positive (15, 81) or a negative (16, 82) signal.

1.3 RpoS-independent, growth phase-dependent gene expression

1.3.1 Project rationale

As detailed above, much of our understanding of *E. coli* responses to non-optimal conditions has relied on two systems: 1) downshift or upshift experiments where exponentially growing bacteria are immediately exposed to a stress condition, or 2) experiments studying gene expression changes between physiological growth transitions like exponential phase to stationary phase transition in different backgrounds. These experiments established RpoS as an important regulator mediating responses to stress conditions as a result of coordinated changes in gene expression and cellular physiology (11, 13, 24). A large emphasis on elucidating genes under the control of RpoS has masked the importance/characterization of stationary phase specific genes that are induced independently of RpoS (i.e. induced in both WT and *rpoS* backgrounds). Many of these genes are known (13, 84-88) but there is a lack of comprehensive profiling of these genes and the number of such genes is expected to be much larger (13). As discussed below, many of these genes and signalling pathways are discovered and characterized in unrelated contexts and have recently gained increasing attention in the fields of biofilm formation and persistence. Perhaps, the phrase "RpoS-independent but growth phase-dependent gene expression" overlooks a complex regulatory cross-talk between regulators like ppGpp, Lrp, H-NS (histone-like nucleoid associated protein), indole, AI-2 (autoinducer-2) that mediate regulated expression of at least a subset of these genes (as detailed above). A comprehensive understanding of the stationary phase and

undiscovered connections between persisters, biofilms, and stationary phase planktonic cells however, requires a comprehensive profiling of these genes. The following is a discussion of some of these genes.

1.3.2 Known RpoS-independent stationary phase-specific genes

rmf (encoding ribosome modulation factor)

Upon transition to stationary phase, translation machinery including ribosomal proteins and associated rRNAs, undergoes reduction in synthesis (89). Under exponential growth conditions, about half of the 50S and 30S subunits combine to form 70S ribosomes. In stationary phase about 40% of the 70S ribosomes are converted to non-active 100S dimers. This is mediated by ribosome modulation factor (90) (encoded by *rmf*), which shows RpoS-independent induction during stationary phase (87). Expression of *rmf* requires ppGpp illustrating the interplay of multiple regulators acting together in single physiological state (66). Rmf is thought to inactivate excess ribosomes during stationary phase and promotes viability during non-optimal conditions like heat stress or osmotic shock (91).

mcbA (a gene for microcin synthesis)

The regulator of stationary phase morphology encoded by *bolA* is under positive control of RpoS. The *bolAp1* promoter contains a "gearbox" element, which is also common to some other stationary phase-specific genes like *mcbA*. Unlike *bolAp1*, however, *mcbA* (a gene of the microcin synthesis operon) is induced upon entry into stationary phase but does not require RpoS for induction (92).

Genes whose products antagonize RpoD function: *ssrS* (encoding 6S RNA) and *rsd* (encoding RpoD anti-sigma factor)

In stationary phase, the concentration of RpoS is lower than the housekeeping sigma factor RpoD and RpoS also has lower affinity for RNAP than RpoD (93). As such, maximal induction of the RpoS regulon is achieved by inhibition of RpoD activity combined with an increase in cellular levels of RpoS. Two regulators, the 6S RNA and Rsd, are growth-phase-dependent but RpoS-independent and inhibit RpoD activity in stationary phase. *rsd* encodes a stationary phase specific anti-sigma factor that binds RpoD (94) resulting in a concomitant increase in expression from RpoS-dependent promoters (95). Interestingly, Rsd levels are high even in exponential phase of growth; however, the inhibitory effects of Rsd are only seen in stationary phase (96). 6S RNA is an abundant RNA regulator that resembles a promoter sequence and therefore causes sequestration of RpoD bound RNAP, downregulating gene expression at a global scale during transition to stationary phase (97). After dilution into rich media, levels of 6S RNA (normalized to levels of 5S RNA) increase from about 1000 copies per cell to 10000 copies by 24 h of incubation with a consistent increase. Furthermore, this regulation is not affected by an *rpoS::*Tn*10* mutation. 6S RNA specifically binds RpoD-RNAP and inhibits its activity without affecting RpoS-RNAP activity (86). Despite the abundance of 6S and given that the majority of RNAP is bound by 6S in stationary phase, not all promoters are affected. A weak -35 element determines promoter specificity at promoters that are upregulated or downregulated by 6S RNA. During late stationary phase (16-24 h of incubation), a deletion of *ssrS* has global effects on mRNA levels

although the fold-changes are relatively small (98). Another study tested the effect of *ssrS* deletion on global transcription during exponential (OD600 = 0.6) and early stationary phases (OD600 = 2.4) phases during growth in YT (yeast extract and tryptone) medium. In exponential phase *guaD-ygfQ* operon (encoding a guanine deaminase and a transporter, respectively) and *tdcABCDEFG* operon (encoding serine and threonine degradation proteins) are highly repressed by 6S RNA. Interestingly, stress response proteins Dps, UspF, UspG are downregulated in exponential phase. During early stationary phase, proteins that interact with H-NS and bind to DNA (Hha, YdgT, and SlyA) and the tryptophan transporter (Mtr) are downregulated by 6S RNA, while a large number of genes related to translation are upregulated (99).

Genes encoding the universal stress proteins

In *E. coli* universal stress proteins UspA, UspC, UspD, and UspE are induced during entry into stationary phase in an RpoS-independent but ppGpp-dependent manner (100). UspA is a general stress response protein induced in conditions that cause reduction in growth rate. UspC, UspD, and UspE are paralogs of UspA that play non-redundant roles and are regulated similarly: all proteins are induced during glucose, phosphate, and nitrogen limitation and also during treatment with mitocyin C. Single deletion mutants in these genes have reduced viability compared to the WT when exposed to UV radiation (100).

Other stationary phase-specific genes

Stringent starvation protein A (encoded by *sspA*) is induced independently of RpoS (11) in stationary phase and affects the expression of some proteins during

extended stationary phase. *sspA* is dependent on ppGpp (101). *rob* encodes a transcriptional regulator that affects gene expression in response to antibiotic-stress (102). Expression of *rob* is induced during stationary phase (103). *glgS* is another RpoS-independent but growth phase-dependent gene (11) responsible for synthesizing glycogen during stationary phase (104).

1.3.3 Persistence and biofilms: Indole, AI-2, and toxin anti-toxin modules

Persisters are a small subset of cells in planktonic and biofilm populations with low enough metabolism to survive antibiotic treatment. Persister gene expression is characterized by expression of TA (toxin-antitoxin) modules, stress response genes, and DNA replication inhibitors. Overall transcription profile in persisters resembles exponential phase cells however, energy production functions and flagellar gene expression is repressed. Stationary phase catalase (*katE*) and other stationary phase genes like *bolA* are downregulated in persisters. Toxin-antitoxin systems are upregulated in persister cells with modules *yafQ-dinJ* and *yoeB-yefM* among others (105). The toxin gene *mqsR* is the most highly induced gene in persister cells (105) and is also induced in *Escherichia coli* biofilms (19). MqsR is an mRNA interferase that hydrolyses mRNAs at GC[A/U] in a translation-independent manner. Expression of MqsR is induced during glucose and amino acid starvation but does not require RpoS (106). MqsR overexpression does not lyse the cells but causes a reversible inhibition of growth that is overcome by expression of the anti-toxin MqsA (107).

The positive role of MqsR in promoting persistence occurs through its upregulation of a DNA replication inhibitor and toxin, CspD (108). CspD is also induced

independently of RpoS (88) and causes a filamentous phenotype due to interference with initiation and elongation steps during DNA replication (109). It was recently reported that cAMP-CRP activates the *cspD* promoter *in vivo* and *in vitro* (110). In addition to upregulating the expression of CspD, MqsR also induces Hha, another toxin that increases persister cell formation (111). The corresponding antitoxin MqsA is unique in that it acts as a repressor at a subset of promoters including the *cspD* promoter (108). Additionally, MqsA represses the RpoS-mediated general stress response and c-di-GMP signalling which are necessary to promote adhesion and a non-motile lifestyle. Oxidative stress triggers degradation of MqsA by cellular proteases leading to induction of the general stress response (112) and toxin gene expression through CspD which can trigger persister formation (108). Hence, the toxin-antitoxin operon *mqsRA* plays a critical role in persister formation by mediating global changes in gene expression through RpoS and other regulators (113). In contrast to the toxin MqsR that promotes RpoS-mediated stress response and adhesion, toxin YafQ cleaves *rpoS* transcripts and therefore reduces RpoS signalling. YafQ is an endoribonuclease with recognition sequence 5'-AAA-G/A-3' (114). The increase in persistence upon *yafQ* overexpression is due to cleavage and reduction in tryptophanase mRNA transcript leading to reduction in indole signalling since indole negatively regulates persistence. Consistent with this, overexpression of *yafQ* increases persistence to ampicillin (betalactam) and ciprofloxacin (fluoroquinone) (83).

mqsR is also expressed in biofilms (18) and regulates biofilm formation by mediating signalling between quorum sensing signal autoinducer-2 (AI-2) and motility functions. AI-2 is internalized into the cells during entry into stationary phase (115) and

activates MqsR which then activates motility functions promoting biofilm formation (116). This suggests that signalling occurring in exponential phase cells leads to a pathway that later culminates in biofilm formation providing a temporal link between distinct physiological states. AI-2 internalization and signalling is controlled by cAMP-CRP (117) and AI-2 affects gene expression in a global manner (118). Decrease in AI-2 extracellular concentrations observed during entry into stationary phase could be due to internalization or due to presence of additional stationary phase signals that reduce AI-2 concentrations (119).

In addition to AI-2 signalling during exponential phase, indole is a signalling molecule that accumulates in the culture supernatant during entry into stationary phase in an RpoS-independent manner (this study). In planktonic cells, indole induces the expression of multidrug exporter genes (79) (*mdtA*, *mdtE*, *emrK*), acid resistance genes (80) (*gadABC*, *cadABC*), regulates cell division (77, 120), and long-term growth and cell viability (75). The idea that signalling pathways occurring during fast growth (AI-2 and indole signalling during exponential and early stationary phase respectively), play an important role in mature cultures, biofilms, and persisters is further supported by the role of indole in biofilm formation (16, 82, 121) and persistence (122). Temporal expression of additional regulators may play a role in mediating signalling pathways in later stages of growth. For example, *yliH* (*bssR*) encodes an RpoS-independent biofilm regulator that becomes highly abundant (at the transcript level) in a 24 h culture (this work and (123)) and is also highly induced in biofilms (16). Deletion of *bssR* increases biofilm formation in LB-glucose. BssR affects gene expression in a global manner by reducing intracellular

and extracellular indole signalling and reducing extracellular AI-2 levels and by repressing motility related genes (16).

Based on the above discussion, Figure 1 summarizes major changes occurring in a batch culture as the cells transition from exponential to stationary phase and until net growth stops in late stationary phase. During exponential phase, RpoS levels are relatively low while flagallar genes are expressed. Extracellular concentration of AI-2 is also high in the growth medium. During transition to stationary phase, RpoS regulon is induced and RpoS negatively regulates flagellar gene expression. ppGpp, which induces *rpoS*, is also synthesized by the cell. These changes lead to reduction in promoter activity of stable RNA operons like the ribosomal RNA operons. Indole is produced by TnaA during stationary phase. Gene expression during extended incubation is understudied. The data presented in this study suggests that while RpoS is mainly active during early stationary phase, a set of RpoS-independent but stationary phase specific genes become relatively important following early stationary phase adaptation (figure 1).

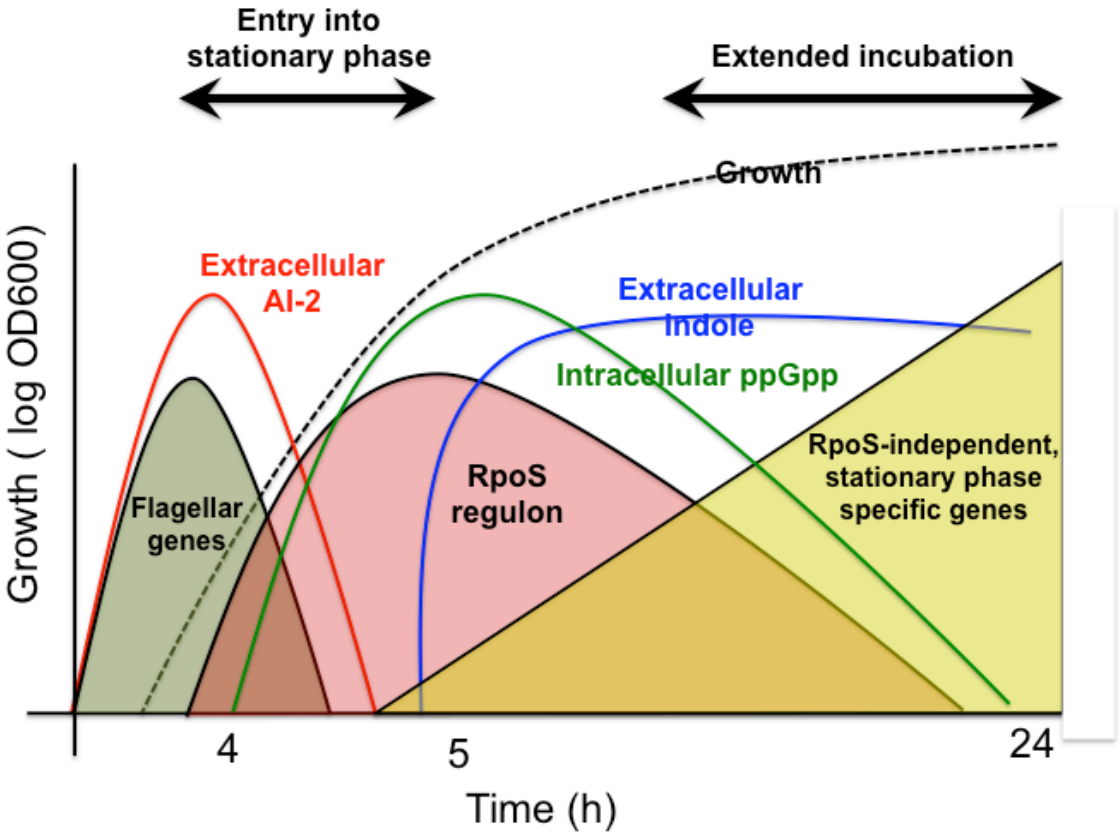

Figure 1: Major changes in the levels/expression of regulators and sets of genes during growth in a batch culture.

Chapter 2: Unbiased screen for long-term stationary phase-active promoters

Preface

The GFP experiments were conducted with Vivan Ly, a former undergraduate thesis student.

2.0 Introduction

To identify promoters with continued activity during extended incubation in rich medium (upto 7 days after which the promoter activity stopped), a library of 1920 promoters in *E. coli* fused upstream of a fast-folding variant of GFP (encoded by *gfpmut2*) on a low copy plasmid pUA66 (figure 2) was employed. **The library was made by the group referenced in (124).**

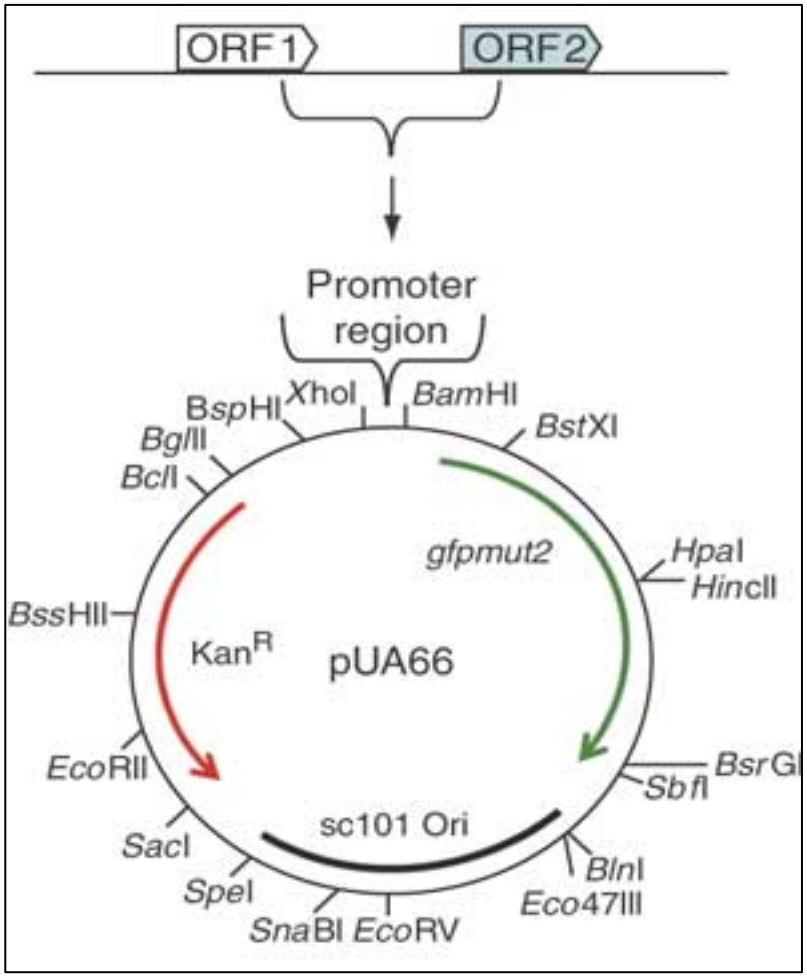

Figure 2: Schematic of the reporter plasmid used for screening of long-term stationary phase-active promoters.

Intergenic regions extending 50 to 150 bp into the flanking coding regions were PCR amplified and cloned between the *X*hoI and *Bam*HI sites upstream of a strong ribosome-binding site in the pUA66 plasmid. The plasmid contains the *gfpmut2* gene, encoding a fast-folding variant of GFP as a reporter and kanamycin resistance. Image from (124).

This system has the advantage that GFP is non-toxic to the cells and does not require ATP for detection unlike luciferase based systems (125). Since GFP is a stable protein, fluorescence is predicted to increase stably as a result of continued promoter activity over time due to accumulation of GFP. Fluorescence was measured at 6 h (early stationary phase), 24 h, 48 h, 120 h, and 168 h following inoculation. Although overnight cultures were grown in kanamycin to maintain plasmid, the antibiotic was not added after subculturing so as to avoid extraneous effects of oxidative stress on the cells. Time points after early stationary phase were chosen for the screen because our data (see below) suggested that RpoS-activity peaks during early stationary phase and declines during extended incubation when RpoS-independent stationary phase gene expression becomes relatively important. Individual wells were screened for consistent rise in fluorescence after 6 h of incubation upto seven days, after which promoter activity was not detected.

2.1 Materials and Methods

2.1.1 Growth conditions

Strains were streaked from -80 °C glycerol stock cultures onto LB plates with appropriate antibiotics to isolate single colonies. Single colonies were inoculated in LB (Miller, 10 g of peptone, 5 g of yeast extract, and 10 g of NaCl) with appropriate antibiotics (Cm 25 μg/ml, Amp 100 μg/ml, Kan 25 μg/ml) for overnight growth. Overnight cultures were diluted 1:10000 in prewarmed LB and grown at 37 °C with shaking at 200 RPM in 50 ml of LB in 250 ml flasks with aeration. GFP screen for long-term stationary phase-active promoters was conducted in 150 μL LB layered with 100 μL of mineral oil in 96-well microtiter plates as described in (124) to prevent evaporation during extended incubation. Fluorescence was measured using a TECAN Safire plate reader with lid open and excitation from the top of the well. Emission and excitation wavelengths were optimized for largest signal to background ratio using a 3-D scan. Excitation wavelength was 485 nm and emission detection wavelength was 507 nm. For catabolite repression experiments, LB was supplemented with 0.5% of glucose (126). Exponential phase is defined experimentally as a culture at OD600 = 0.3 and early stationary phase at OD600 = 1.5.

One-step gene inactivation protocol was used to inactivate *tnaA* and *clpP* as described by (127). Briefly, primers containing 5' 40-bp homology to the flanking regions of the target genes were used to amplify the chloramphenicol resistance cassette from plasmid pKD3. The PCR product was gel purified and electroporated into *E. coli* K12 MG1655 containing plasmid pKD46, which contains phage λ recombination genes to

facilitate recombination of the PCR product into the genome. Primers flanking the target gene and primers within the target gene were used to confirm deletion. Electrocompetent cells were prepared by washing in ice-cold 10% glycerol three times and concentrating 100-fold.

2.1.2 Protein identification using MALDI-TOF-MS

The protocol for in-gel trypsin digestion was as described by Shevchenko et al (128). Briefly the proteins (see immunoblot procedure for protein preparation) were resolved on 12% polyacrylamide gel and stained with Coomassie Blue dye. The band of interest was excised from the gel and cut into smaller pieces with a sterile scalpel. The pieces were destained with a 1:1 (vol:vol) of acetonitrile and 100 mM ammonium bicarbonate for 30 minutes. The destained gel pieces were dehydrated using acetonitrile and rehydrated with reduction solution (10 mM DTT in 100 mM NH_4CO_4) and incubated at 56 °C for 30 minutes to reduce the disulphide bonds (if any) in the protein(s) contained in the band of interest. The gel pieces were dehydrated with acetonitrile and rehydrated with alkylation solution (55 mM iodoacetamide in 100 mM NH_4CO_4) to prevent reformation of disulphide brides. The gel pieces were dehydrated again and rehydrated with porcine trypsin prepared in NH_4HCO_3 buffer and incubated overnight at 37 °C. The peptides were eluted in 60% acetonitrile using 0.1% trifluoroacetic acid and identified using MALDI-TOF-MS. Peak list was obtained from Biointerfaces Institute, McMaster and protein identity was found using web-based Mascot peptide search engine by inquiring against the Swiss-prot database.

2.1.3 Immunoblot and SDS-PAGE

At experimental time points, samples of the cultures were centrifuged at 14000 g for two minutes. The supernatant was removed and pellets were resuspended in SDS (sodium dodecyl sulphate) loading buffer (2×Laemmli Buffer: 125 mM Tris-Cl, pH 6.8; 2.5% β-mercaptoethanol; 8.7% glycerol; 1% SDS; 0.01% Bromophenol Blue) to a final cell concentration equivalent to an OD600 of 15. The cells were heated at 100°C for 5 min**.** Protein was resolved on polyacrylamide gels and stained with 0.1% Coomassie Blue dye for studying protein profile and to confirm equal loading of sample among time points. For immunoblot analysis, proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Inc., Billerica, MA). The membrane was soaked in blocking solution containing 5% milk in TBS-T buffer (87 mM NaCl; 10 mM Tris-Cl, pH 8; 0.05% Tween 20) for one h at room temperature. The primary RpoS/RpoF/RpoD (Neoclone, Inc., Madison, WI) or TnaA (Neobiolabs Inc., Cambridge, MA) antibodies were added at a dilution of 1:10000 (1:1000 for TnaA antibody) and the membrane was incubated overnight with gentle shaking at 4°C. The membrane was washed three times with TBS-T and incubated for 1 h at room temperature with shaking in a 1:3000 (1:20000 for TnaA anti-rabbit (GE Healthcare Inc., Quebec, Canada)) dilution of the secondary anti-mouse antibody (Bio-Rad, Inc., Mississauga, Ontario). The membrane was washed three times with TBS-T. The secondary antibody fluorophore was activated with ECL staining solution (1:1 detection reagent mixture; Amersham GE Healthcare Inc., Quebec, Canada) for one min. The membrane was exposed to Amersham Hyperfilm ECL for 10 s – one min.

2.1.4 RT-qPCR for gene expression

Total RNA was isolated at exponential phase (OD600 = 0.3) or early stationary phase (OD600 = 1.5) using Norgen Total RNA Purification Kit. Quality of the isolated RNA was checked using agarose gel electrophoresis and by measuring absorbance 260/280 ratio using a Nanodrop 2000 (Thermo scientific). RNA was DNase treated and re-purified using RNA clean and concentrator kit (Zymo Research). Five hundred nanograms of RNA was reverse transcribed to cDNA with random hexamer primers using iScript cDNA Synthesis Kit (Biorad). mRNA levels were quantified using SsoFast Evagreen Supermix and CFX-96 Real Time PCR system. 16S ribosomal RNA (*rrsA*) was used as a reference gene for normalization. Specificity and efficiency of amplification of each primer pair was verified by constructing a standard curve of amplification using purified *E. coli* genomic DNA template.

2.1.5 Kovacs Assay for indole quantification

One milliliter of culture was centrifuged at 14000 g at two minutes and the supernatant was removed. Three hundred microliters of Kovac's reagent was added to the supernatant for two minutes. A dark purple organic product is produced. Hundred microliters of the organic reaction product was diluted into 900 µL of HCl-amyl alcohol. Absorbance at 545 nm was measured to quantify extracellular indole concentration against a reference curve.

2.2 Results and discussion

Individual wells were screened for promoters active after growth senescence which occurred between six and 24 h of incubation. Promoters with low activity (< ~3-fold RFU (relative fluorescence units) above background measured from the promoterless strain) were not selected. The selected promoters on average had a 9-fold increase in fluorescence between early stationary phase (6 h) and 7 days and showed a consistent increase during prolonged incubation at later times indicative of continued promoter activity. Table 1 shows two examples of promoters selected for long-term stationary phase activity (controlling expression of *rmf* and *mqsR*) and a promoter that was not active during stationary phase (controlling *ribC*).

Table 1: Examples of rejected promoters and promoters identified as active during extended incubation.
For a promoter to be selected, fluorescence at any time during extended incubation had to be more than at least three-fold above the background as measured from the promoterless strain and had to show continued expression (measured by increase in fluorescence normalized to OD600). The following numbers are background subtracted RFU normalized to OD600.

Time (h)	**Promoterless strain (background)**	**Promoter not active during extended growth (*ribC*)**	**Relatively strong and a growth phase-dependent promoter (*rmf*)**	**Relatively weak but growth phase-dependent promoter (*mqsR*)**
5	96	747	2609	1064
24	214	970	12200	2902
48	139	1189	27282	4294
120	267	1414	46148	6505
168	325	1107	49571	6081

Two hundred and twenty nine selected genes fell into major categories, based on molecular function of catalytic activity, DNA binding, transport, and transcription factor activity. Out of the 229 selected promoters, only 35 were positively-dependent on RpoS

and 15 were negatively-dependent during early stationary phase (cross-comparison with published microarray datasets). The rest of the promoters were RpoS-independent but growth phase-dependent. The following is a discussion of genes of interest and their physiological function with relevant references and information from the Ecocyc database (complete list of the genes and their associated promoter activity during long-term incubation can be found in the appendix).

2.2.1 Promoters of interest identified: Toxin-antitoxin genes

Two toxin-antitoxin systems *mqsRA* (and its effector gene *cspD*, a DNA replication inhibitor) and *yafQ-dinJ* were identified as genes expressed after growth senescence (figure 3). The role of these genes in persistence and biofilms has emerged relatively recently (108).

mqsR is the most highly-induced gene in persister cells (105) and is also induced in biofilms (18). MqsR toxin is an activator of DNA replication inhibitor gene *cspD* (108) which itself is induced during stationary phase (88). Growth and viability are reduced upon CspD production. CspD expression causes a filamentous phenotype due to interference with initiation and elongation steps during DNA replication (109). Activation of MqsR during stress conditions leads to increasing levels of CspD and Hha leading to persistence (111). The antitoxin MqsA, in addition to repressing transcription of its own operon, represses RpoS-mediated general stress response and c-di-GMP signalling, responsible for promoting adhesion. MqsA, therefore, promotes motility (113). Unless, MqsA is actively degraded in long-term cultures, continued expression of *mqsRA* is predicted to cause repression of *rpoS* transcription. Repression of the general stress

response may itself lead to further oxidative damage in the cell and activation of proteases, which cleave MqsA leading to persister formation. Furthermore, it can be speculated that this pathway may operate only in a subset of cells in the population (that form persisters) that have exceeded damage beyond a certain threshold. Growth phase-dependent increase in *mqsRA* transcription predicts that the number of persisters increase in long-term stationary phase due to increased MqsR if MqsA is inactivated (figure 4). Indeed, we observe that RpoS activity peaks during early stationary phase and RpoS transcript levels drop to exponential phase levels after 24 h of incubation (see below). YafQ is an endoribonuclease with mRNA transcript recognition sequence 5'-AAA-G/A-3' (114). Inactivation *rpoS* and *tnaA* transcripts by YafQ results in reduction in RpoS-mediated general stress response and indole signalling leading to persistence since indole negatively regulates persistence (83). Promoter activity from the toxin-antitoxin genes is shown in figure 3.

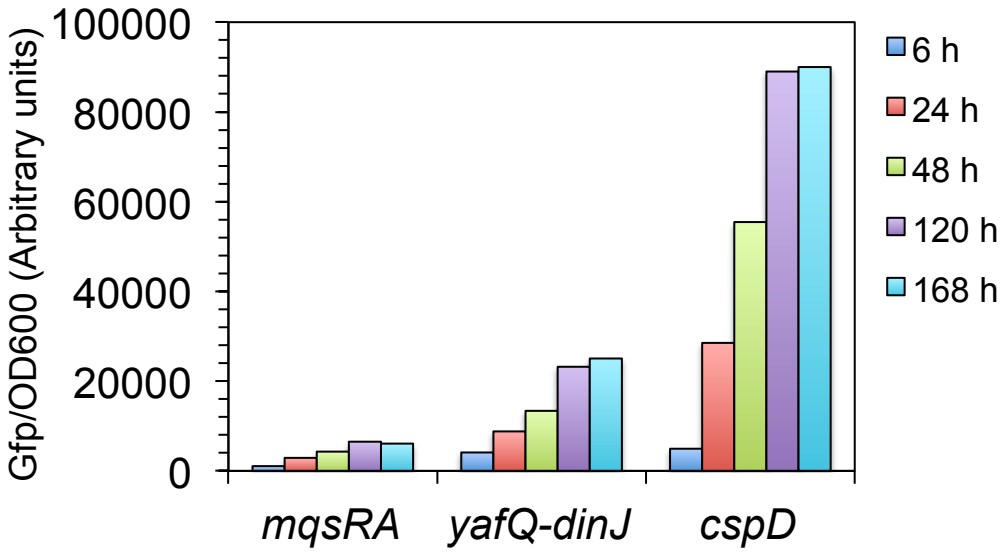

Figure 3: Long-term promoter activity of two toxin-antitoxin modules *mqsRA* and *yafQ-dinJ* and replication inhibitor *cspD*.

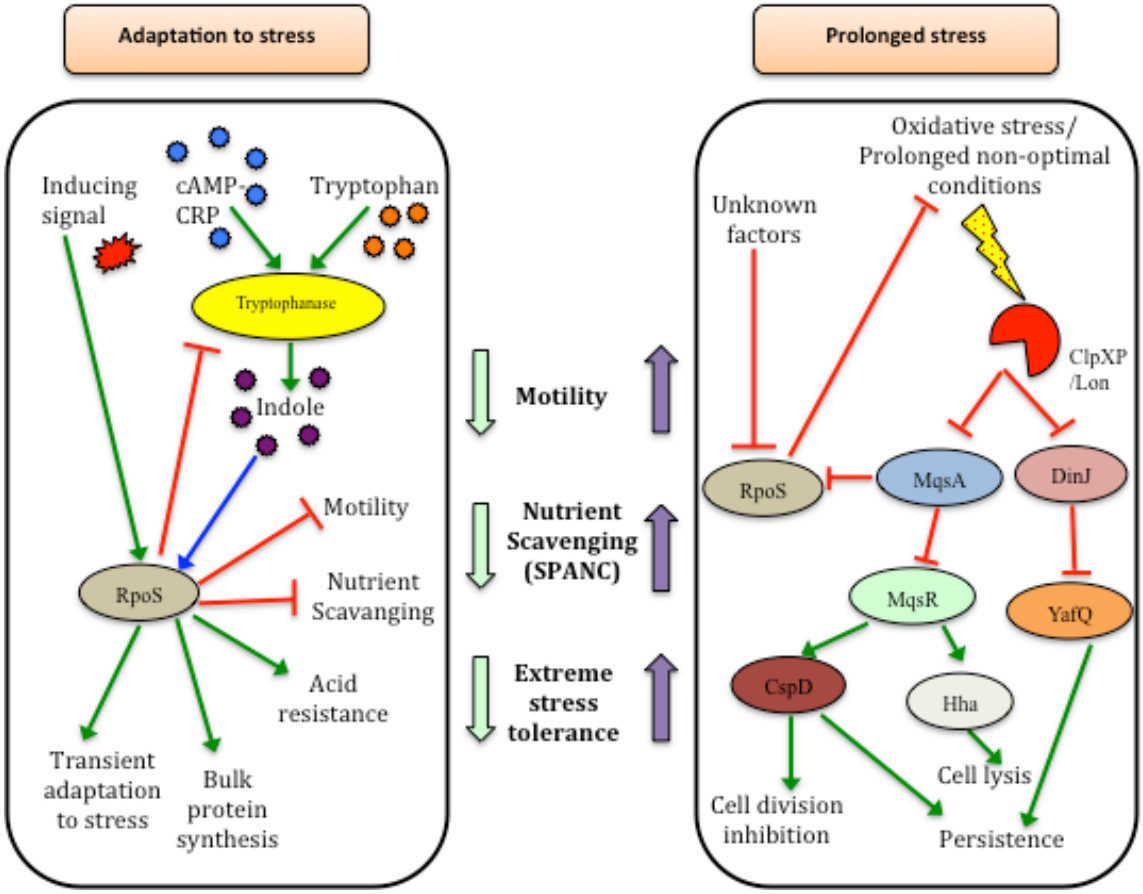

Figure 4: A predicted model for cessation of RpoS activity and increased frequency of antibiotic tolerant cells in long-term starved cultures.

Such a pathway may operate in a subset of cells leading to heterogeneous population structure. Activated MqsR represses RpoS activity leading to further decrease in environmental fitness and persistence. The effect of indole on RpoS activity occurs during stationary phase and is not completely understood (see the section on tryptophanase below). SPANC (Self-Preservation And Nutritional Competence) refers to the trade-off between expression of metabolically costly stress defense genes and nutrient scavenging genes.

2.2.2 Promoters of interest identified: RpoS-independent stress response genes

Stringent starvation protein A (encoded by *sspA*) is a stationary phase specific gene induced during glucose limitation, amino acid limitation, and reduction in growth rate. Proteins synthesized during extended stationary phase (24 h – 72 h) are different in the WT and *sspA* backgrounds and expression of *sspA* is dependent on ppGpp (101) and not on RpoS (11). During entry into stationary phase, ribosome activity is reduced by conversion of 70S ribosomes to 100S dimers. This is facilitated by ribosome modulation factor, which showed continued expression during extended incubation. Rmf is induced during entry into stationary phase independently of RpoS (87, 90, 91). In stationary phase, both the concentration and affinity of RpoS for RNAP are lower than the housekeeping sigma factor RpoD (93). *rsd* encodes an anti-sigma factor that inhibits RpoD function to facilitate RpoS function. Expression of *rsd* is stationary phase specific (93) and RpoS-independent (11). Universal stress protein E (UspE) was induced during late incubation in this study. UspE is induced during entry into stationary phase (100) and oxidative stress (129) and negatively regulates biofilm formation (129). *uspF*, another locus identified in this screen (figure 5), plays an opposite role of promoting motility (84) and shows stationary phase induction. *uspF* is also negatively regulated by RpoS during early stationary phase (11). *rob* encodes a transcriptional positive regulator that has high sequence similarity to the *marA* and *soxS* gene products and activates a subset of genes in response to antibiotics and redox-cycling compounds (102). Expression of *rob* increases upon entry into stationary phase in rich medium, during glucose and phosphate limitation in minimal medium, and expression of a set of proteins is altered in the *rob* null mutant

during extended stationary phase (103). Expression of *glgS* continued during extended incubation and this induction is independent of RpoS (11). GlgS synthesizes glycogen during stationary phase and reduces biofilm formation and motility (104). *yobF* encodes a small protein responsible for membrane integrity and is induced in stationary phase and post heat-shock in an RpoS-independent manner (130, 131). Expression of several cold-shock induced proteins was also seen during extended incubation. These include *lpxP* (codes for an enzyme that incorporates palmitoleate into lipid A molecules (132)), cold-shock protein B (133), and cold-shock protein A (134).

Error prone repair of DNA double strand breaks under stress conditions results in stress-induced mutagenesis. *cyoA*, which encodes a subunit of cytochrome oxidase acts upstream of sigma factors RpoS and RpoE in promoting stress induced mutagenesis. Other genes identified in this screen that play a role in stress induced mutagenesis are *yifE* and *ligA* (135). Poly A polymerase 1 (PAP 1) polyadenylates about 90% of the mRNA's in exponential phase that do not require the Rho factor for transcription termination (136). A large number of *E. coli* transcripts undergo polyadenylation and expression of PAP 1 is inversely correlated with growth rate. Late expression of PAP 1 as observed here may lead to differential mRNA stability under these growth conditions (137). GatY is a subunit of the tagatose-1, 6-bisphosphate aldolase complex, the expression of which is induced upon stress conditions and during growth in acetate, the same conditions that induce RpoS (138). In addition to promoting mutagenesis and regulating mRNA half-lives, cells in long-term stationary phase may also turnover misfolded or aberrant proteins. *hslV* which forms the peptidase subunit of the heat shock sigma factor induced

protease (139, 140) was expressed during extended incubation in this study. Another long-term expression gene *htpX* codes for a protease that degrades misfolded proteins (141). The extracytoplasmic sigma factor, RpoE, which controls regulon members mediating the response to envelope stress and protein misfolding, was also induced (142). While continued incubation under batch culture conditions ultimately leads to exhaustion of carbon and nitrogen sources, limitation of ions and other minerals necessary to support the function of certain enzymes probably occurs. Mg^{2+} ion limitation is sensed by the cell through the PhoP-PhoQ two component systems, which activate downstream targets including the RstA-RstB two component system. *rstAB* operon was strongly growth phase-dependent. *slyB* (an outer membrane protein) and *yrbL* (uncharacterized protein) are two other genes identified in this study that form components of the Mg^{2+} stimulon (143).

During entry into stationary phase, *E. coli* produces a signalling molecule indole by conversion of exogenous tryptophan to indole, pyruvate, and ammonia (144). *tnaA* is an RpoS-independent but stationary phase specific gene with complex regulation, which is more fully studied in this report (refer to the section on tryptophanase). Interestingly, TnaA is one of the most induced proteins during alkaline stress in complex media during prolonged incubation (145), conditions that are similar to prolonged growth in non-buffered LB. GatY, a subunit of the tagatose-1, 6-bisphosphate aldolase complex, is another protein identified in this screen that shows accumulation in response to pH stress (145). In this study, *mtr*, which encodes a tryptophan specific importer, also had continued promoter activity during long-term incubation (146).

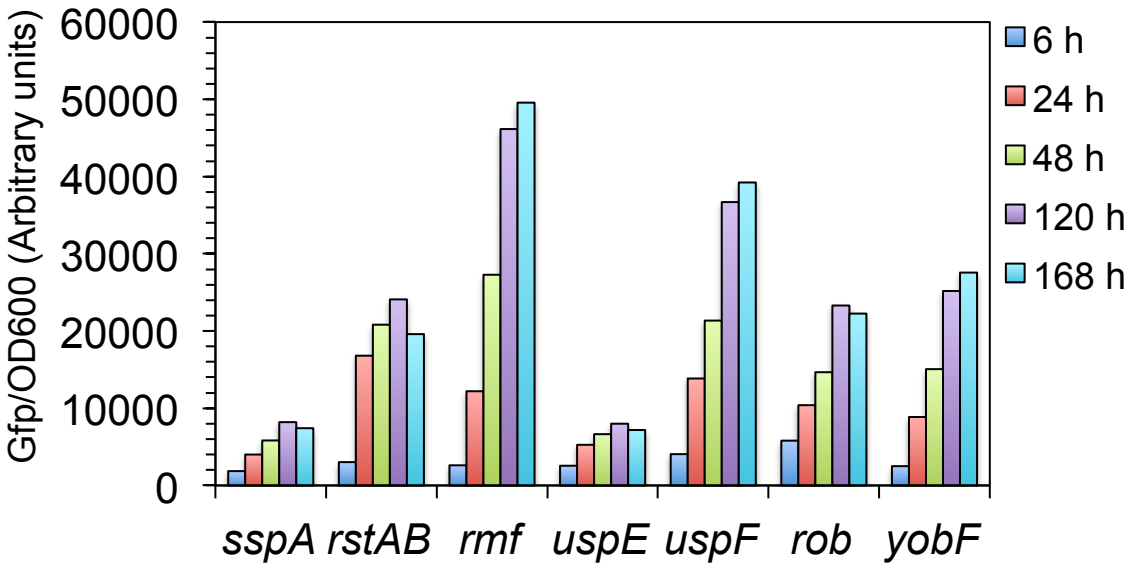

Figure 5: Representative RpoS-independent stress response genes identified in this study.

2.2.3 Promoters of interest identified: Metabolism and biosynthesis

Histidine and branched chain amino acid biosynthesis operons were active during extended incubation suggesting that the cell senses amino acid starvation and retains anabolic functions (figure 6). Expression of the histidine biosynthesis genes is induced by ppGpp (147). Additionally, isoleucine biosynthesis operon was also induced (figure 6) Consistently, *pheA* (encoding chorismate mutase), an enzyme that catalyses the first shared step of tyrosine and phenylalanine biosynthetic pathways (148), was expressed during extended incubation. Both shikimate kinase genes (*aroL* and *aroK*) that also participate in aromatic amino acid biosynthesis were also induced. *dapA* (encoding dihydrodipicolinate synthase) encodes the enzyme that catalyzes the first step of lysine biosynthesis. Expression of *dapA* was also observed during extended incubation (149). Additionally serine biosynthesis (*serA, glyA*) genes were also induced. Interestingly, *pck*, coding for phosphoenolpyruvate carboxykinase, is induced during stationary phase in an *rpoS*-independent manner (150, 151) and is also induced during growth in acetate (152). In addition to biosynthesis, genes for the utilization of arginine (153) (*ast* operon) had continued expression in long-term stationary phase. Enzymes of the pentose phosphate pathway also showed continued expression. Both transaldolase/transketolase operons (*talA-tktB* and *talB-tktA*) showed continued expression in addition to the constitutive ribose phosphate isomerase (154) (*rpiA*) and phospho glucose isomerase (*pgi*). Serine hydroxymethyltransferase (*glyA*) catalyses the biosynthesis of 5, 10 m-tetrahydrofolate which is required for lipid biosynthesis (155). *plsB* encodes an essential enzyme of the

phospholipid biosynthesis pathway and interestingly contributes to persister cell formation (156).

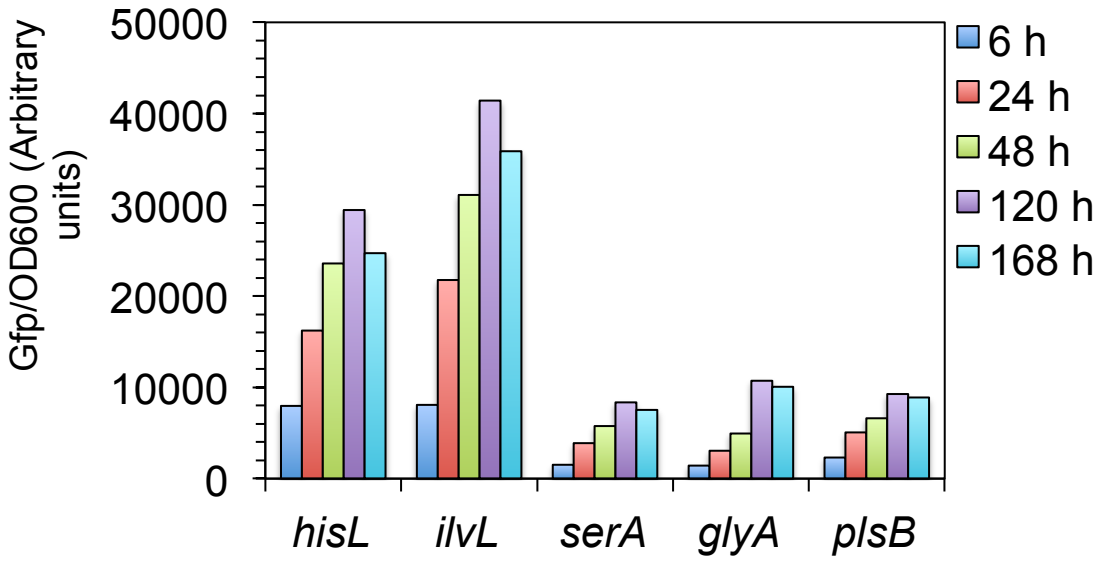

Figure 6: Continued expression of metabolic genes in long-term stationary phase.

2.2.4 Promoters of interest identified: Cryptic prophage genes

icd codes for isocitrate dehydrogenase enzyme, the C-terminus of which is within the cryptic prophage e14 (157) and the product of *icd* contributes to resistance against hydrogen peroxide (158). Hha is a DNA-binding regulator that forms heterodimers with H-NS and induces the expression of some cryptic prophage genes leading to cell lysis and dispersal of biofilms (158). *ybcW* encodes a gene of the DLPD-12 prophage that is positively regulated by Hha (158) and shows continued promoter activity in this study. *ykfG* is a gene of unknown function within the CP4-6 prophage. *cspB* encodes a cold-shock protein on the Qin prophage that interacts with single-stranded RNA in a sequence-specific manner to promote translation and peptide folding (figure 7) (159).

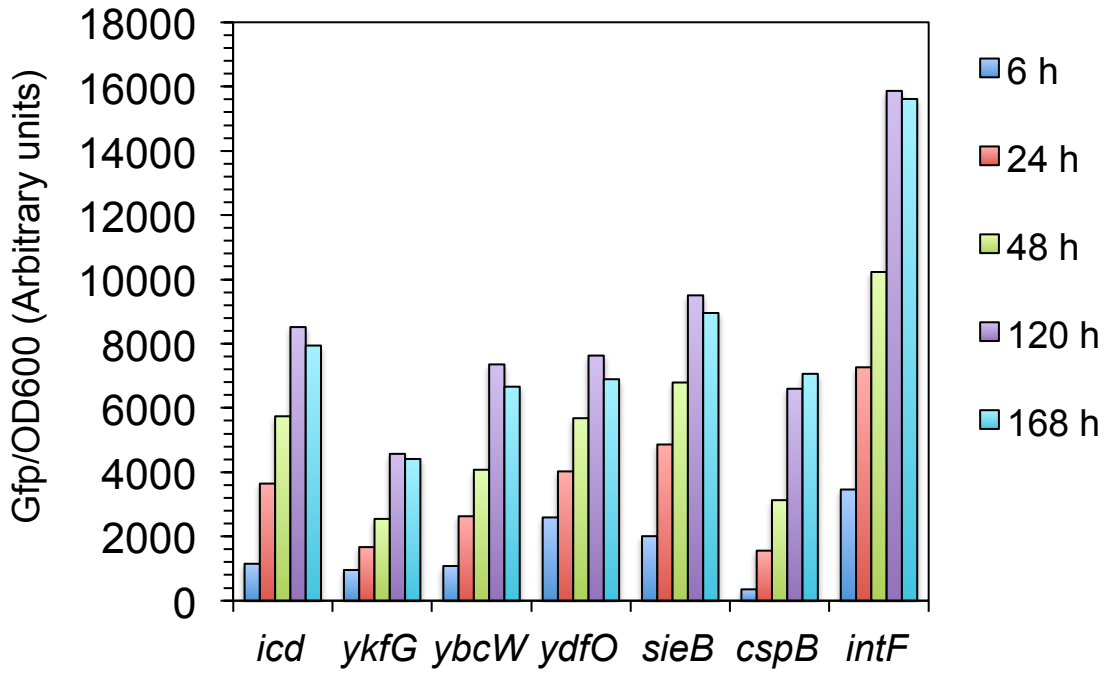

Figure 7: Increase in expression of cryptic prophage genes active during extended incubation.

On the basis of gene function, other genes with continued long-term expression belonged to transporters (*yegT, yhiN, yjjK, artP, mglB, dppA, oppA, ydcS, sstT, pitB, ydjF*), regulators (*yaaH, yeaT, exuR, yedW, gadW, fucR, glcC, mhpR, yifE, yhaJ, uxuR, cytR, gadX, fur, araC, yhiH, ycgE*), and DNA replication inhibitors (*minC, hda*).

2.2.5 RpoS-independent repression of motility in stationary phase

RpoS mediated repression of motility during growth in minimal medium (44) and rich medium (11) conditions is known. In order to test for the effects of *rpoS* deletion on the flagellar sigma factor, RpoF, immunoblot analysis was used. RpoS negatively regulates RpoF levels during long-term stationary phase however, RpoF levels decrease in the *rpoS* mutant strain during extended stationary phase (figure 8). Levels of RpoD, the housekeeping sigma factor, are stable during all growth stages in both strains (figure 8). This suggests that yet unidentified, RpoS-independent factors, cause cessation of flagella mediated motility upon entry into stationary phase.

Figure 8: RpoS-independent repression of flagellar sigma factor RpoF in *E. coli* K12 MG1655

In the presence of RpoS, RpoF levels are substantially reduced during extended incubation however, levels of RpoF are also reduced in the *rpoS* mutant background during extended incubation. The levels of RpoD, the housekeeping sigma factor, are not altered in an *rpoS* null mutant and stable throughout all growth stages.

2.2.6 Role of RpoS during extended incubation

As discussed above, RpoS is induced during entry into stationary phase (induction begins at an OD600 of about 0.3) and regulates gene expression both positively and negatively (9-11, 13, 41). These are conditions of relatively fast growth and complete cessation of growth occurs later during stationary phase (in our lab this corresponds to about 14 – 16 hours of growth when culture density reaches a maximum). Surprisingly 14 genes that showed continued expression during extended incubation (this study) are negatively regulated by RpoS during early stationary phase (11). These were *hisL* (histidine biosynthetic operon leader peptide), *atpI* (a membrane bound ATP synthase), *dmsA* (DMSO reductase), *fucR* (transcriptional activator of L-fucose utilization genes), *aspA* (aspartate ammonia lyase), *hupB* (DNA-binding protein), *hptX* (heat-shock induced protein), *yafK* (an uncharacterized protein), *pck* (phosphoenolpyruvate carboxykinase), *uspE* (universal stress protein E), *uspF* (universal stress protein F), *mtr* (tryptophan specific transporter), *ompC* (an abundant outer membrane protein), *menG* (a methyl-transferase). Another regulator, BssR, plays a crucial role in biofilm formation by mediating complex signalling pathways related to AI-2 and indole signalling (16). Interestingly, *bssR* is highly negatively regulated gene by RpoS during early stationary phase (11) however, *bssR* transcript increased about 3600-fold between early stationary phase and 24 h of incubation in LB – the same time period during which *rpoS* transcript decreased to exponential phase levels (this study, (123), figure 9 and 10).

These findings suggest that the overall RpoS activity is substantially reduced after initial stationary phase adaptation even though many genes continue to increase in

expression. Consistent with this, mRNA levels of three prototypical RpoS-dependent transcripts decrease during extended incubation as measured by reverse transcription – qPCR (figure 9). The reduced expression of RpoS regulon members during extended incubation could be due to decreased levels of the sigma factor itself or modification of its activity. Support for the latter comes from the observation that indole affects RpoS activity at the *gadA/B* promoter (see the section on tryptophanase below) and from the observation that RpoS levels remain high in *E. coli* K12 MG1655 during extended incubation (figure 11). RpoS is highly-unstable, due to ClpXP (160) mediated proteolysis during exponential to early stationary phase – the period when RpoS activity peaks. Reduction of RpoS-dependent gene expression during extended incubation is caused by reduced RpoS activity rather than decreasing levels of the sigma factor which is supported by the finding that RpoS is relatively stable in mature cultures even after addition of chloramphenicol (figure 11). Anti-adapters IraP, IraM, IraD are known to stabilize RpoS in stationary phase and can possibly explain the relative increase in stability during extended incubation (161, 162). Contrary to the expression pattern of *rpoS* in the K12 MG1655 laboratory strain, environmental isolates have relatively higher levels during exponential phase, peak levels during early stationary phase, and decline during long term incubation (figure 19). We cannot conclude that expression of all RpoS regulon members is reduced during long-term growth or that a subset of RpoS-controlled promoters have a role during extended incubation. Supporting the latter is the observation that some RpoS-controlled promoters like *talA*, *elaB, hdeA* have continued activity during the extended incubation – GFP screen. Nonetheless, current data were consistent with the

idea that RpoS is mainly active during early stationary phase and that other regulators become important later after the adaptation period. As discussed above, these include the toxin-antitoxin modules, other stress-response genes, prophage genes, and metabolism genes. Furthermore, attenuation of RpoS activity in mature cultures might be a direct consequence of expression of toxin-antitoxin operons like *mqsRA* and *yafQ-dinJ* (see above).

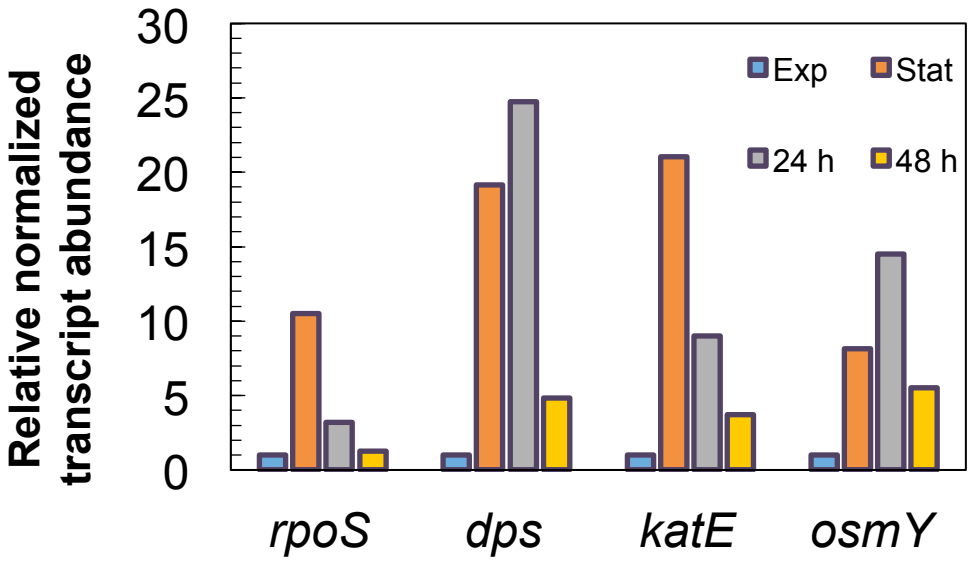

Figure 9: Transcript abundance of *rpoS, dps, katE,* and *osmY* in *E. coli* K12 MG1655 in exponential (OD600 = 0.3), early stationary phase (OD600 = 1.5), 24 h post inoculation, and 48 h post inoculation using RT-qPCR.

16S ribosomal RNA gene (*rrsA*) was used for normalization and results are shown for a single biological replicate with two technical replicates within each strain and experimental time point.

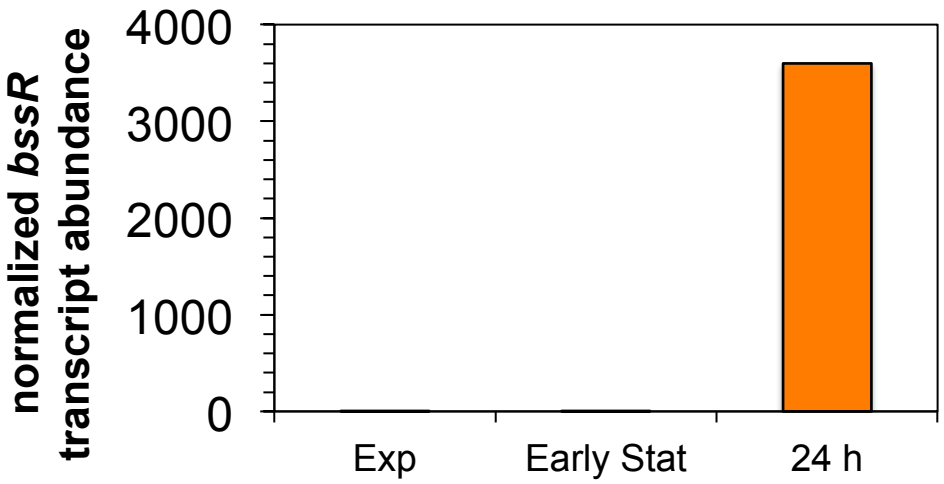

Figure 10: Transcript abundance of *yliH/bssR* in *E. coli* K12 MG1655 in exponential (OD600 = 0.3), early stationary phase (OD600 = 1.5), and 24 h post inoculation using RT-qPCR.

16S ribosomal RNA gene (*rrsA*) was used for normalization and results are shown for a single biological replicate with two technical replicates within each strain and experimental time point.

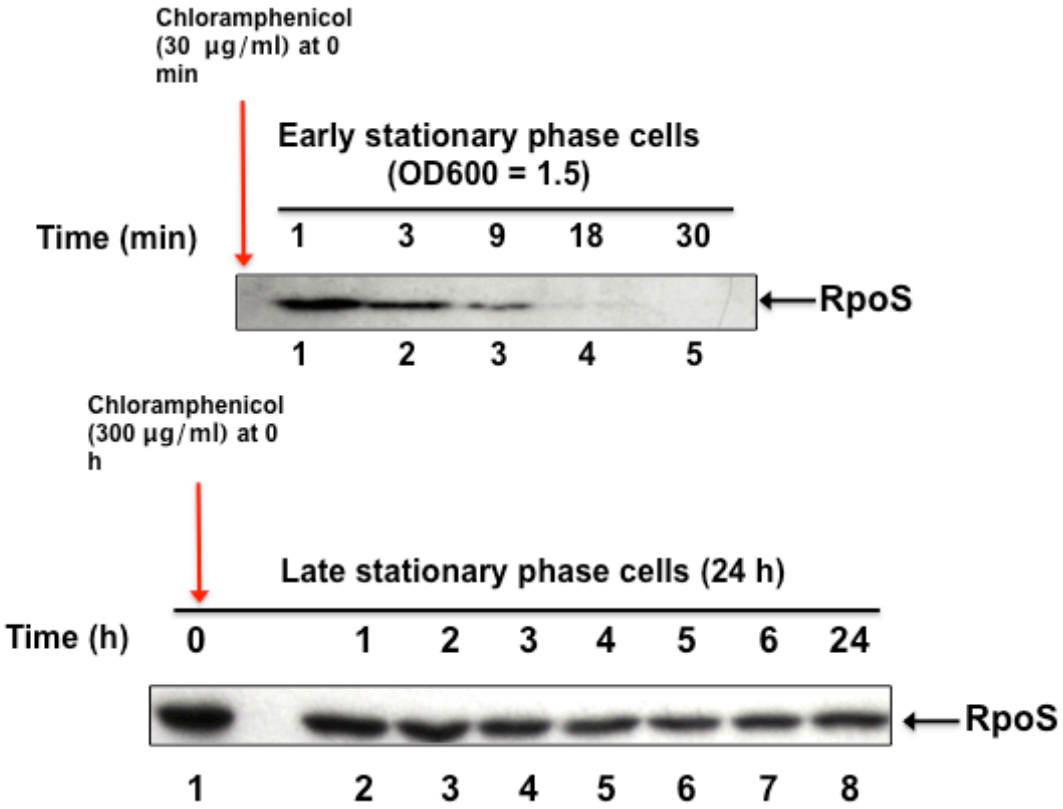

Figure 11: Stability of RpoS after chloramphenicol addition in early stationary phase compared to extended incubation.

Top: 30 µg/ml chloramphenicol was added during early stationary phase (OD600 = 1.5). Bottom: 300 µg/ml chloramphenicol was added after 24 h of incubation and cells were then further sampled to detect RpoS levels upto 24 h following chloramphenicol addition.

2.2.7 Levels of major proteins as a function of incubation time

At the protein level, elongation factor Tu (TufA), the outer membrane proteins OmpC and OmpA are expressed at a stable level throughout all growth stages suggesting that the cell maintains a constant level of these proteins. On the other hand Dps and GadA are stationary phase specific and were stable after initial induction (figure 12). OmpW is a protein under negative regulation by RpoS (11) and was induced in a growth phase-dependent manner only in the *rpoS* background (data not shown).

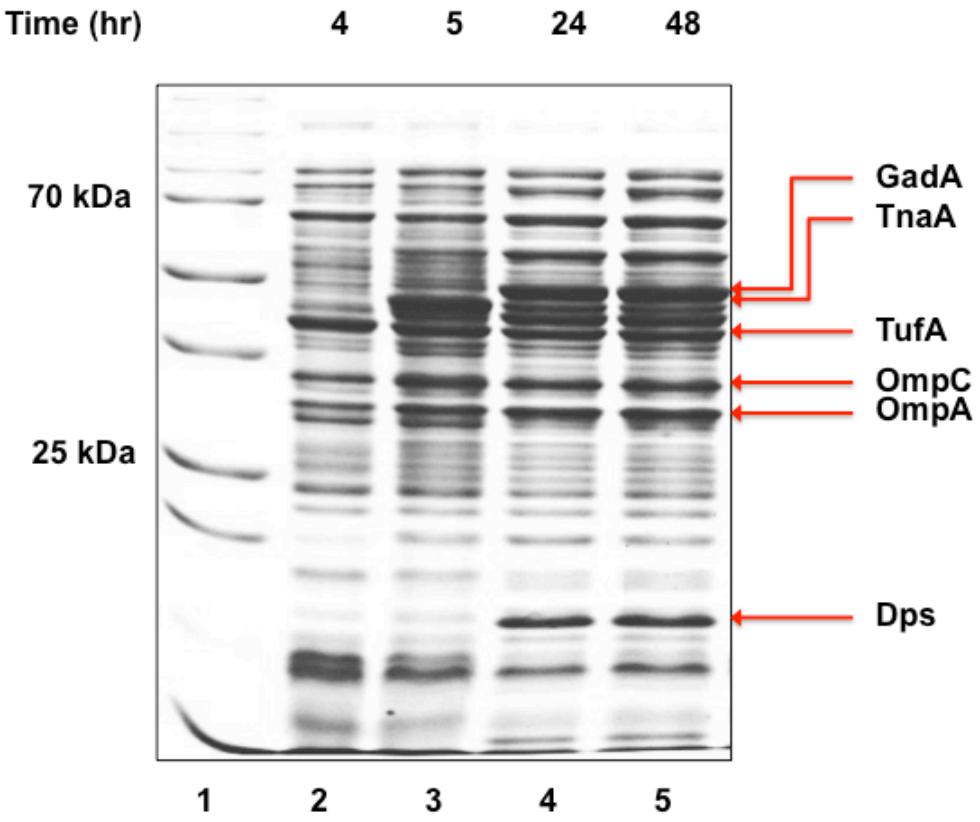

Figure 12: Cellular levels of major proteins as a function of incubation time.

These proteins were identified using MALDI-TOF mass spectrometry of tryptic digests of the bands indicated and search against Swiss-prot database. The image shows SDS-Page gel stained with Coomassie Dye. Equal protein was loaded in each lane.

Chapter 3: The special case of tryptophanase: an RpoS-independent, stationary phase gene

Preface

The *clpP* mutant was isolated along with William Yang, a former undergraduate thesis student.

3.0 Introduction

High levels of a late stationary phase promoter for *tnaCAB* operon encoding tryptophanase and a tryptophan importer were identified in this screen, the regulation of which was further studied at the protein and transcript level. Given the widespread importance of indole as a signalling molecule, it is not surprising that TnaA is subject to complex regulation (refer to discussion of indole above). Transcription of the *tnaC-tnaA-tnaB* operon is dependent on translation of the leader peptide encoded by *tnaC* (144, 163, 164). Maximal induction of tryptophanase is achieved in proportion to exogenous tryptophan and subject to catabolite repression (165). In addition to activation by tryptophan and cAMP-CRP, evidence suggests that indole may be autoregulatory since it activates the expression of *tnaB* (166). At the transcript level, we have previously found that Crl and RpoS positively regulate *tnaA* expression during exponential phase (OD600 = 0.3) (45) but RpoS negatively regulates *tnaA* expression during early stationary phase (OD600 = 1.5) (11). Furthermore, in pathogenic *E. coli* O157:H7, RpoS positively regulates TnaA during early stationary phase (OD600 = 1.5) (9). This work was done to understand the role of RpoS in controlling *tnaA* expression and indole signalling. At the protein level, tryptophanase is induced upon entry into stationary phase in both WT and *rpoS* cells. RpoS attenuates the expression of tryptophanase following this initial induction resulting in transient high levels of TnaA. At the transcript level our findings

confirm that, RpoS can act as either a positive or a negative regulator of *tnaA* expression depending on the growth stage. TnaA regulation was similar among the K12 MG1655 and non-laboratory environmental isolates tested in this study (167), except that indole signalling appeared delayed in these strains and tryptophanase levels were lower. These results suggest that the major mechanism of tryptophanase induction during entry into stationary phase is independent of RpoS however, RpoS modulates TnaA protein levels, highlighting its role as a negative regulator at physiological conditions.

3.1 Results

3.1.1 Tryptophanase is induced during stationary phase entry and RpoS negatively regulates TnaA protein levels

In search for stationary phase specific but RpoS-independent proteins, TnaA was identified as a highly expressed protein upon entry into stationary phase (between OD600 = 0.3 and 1.5) in both WT and *rpoS* backgrounds using in-gel tryptic digestion and MALDI-TOF mass spectrometry as described in Materials and Methods (see also (83)). The identity of the protein was subsequently confirmed by creation of a *tnaA* deletion mutant in which this induced, highly-expressed protein was abolished (figure 13). Interestingly, at the transcript level, we have observed both positive (9, 45) and negative (11) regulation of TnaA by RpoS depending on the growth stage or strains used, which led us to further examine tryptophanase regulation by RpoS at the protein and transcript level in laboratory strains and in a collection of previously-characterized feral environmental isolates. Tryptophanase levels were higher in the *rpoS* background at early stationary phase than the WT strain. Furthermore, TnaA expression was transient only in

the WT background whereas TnaA levels were consistently higher in the *rpoS* background during late stationary phase up to 24 h (figure 13). Indole production in both strains began at an OD600 of about 0.7 (late exponential phase) and reached similar levels after culture saturation at 24 h (figure 13) suggesting that conversion of exogenous tryptophan to indole is complete and equivalent in both strains.

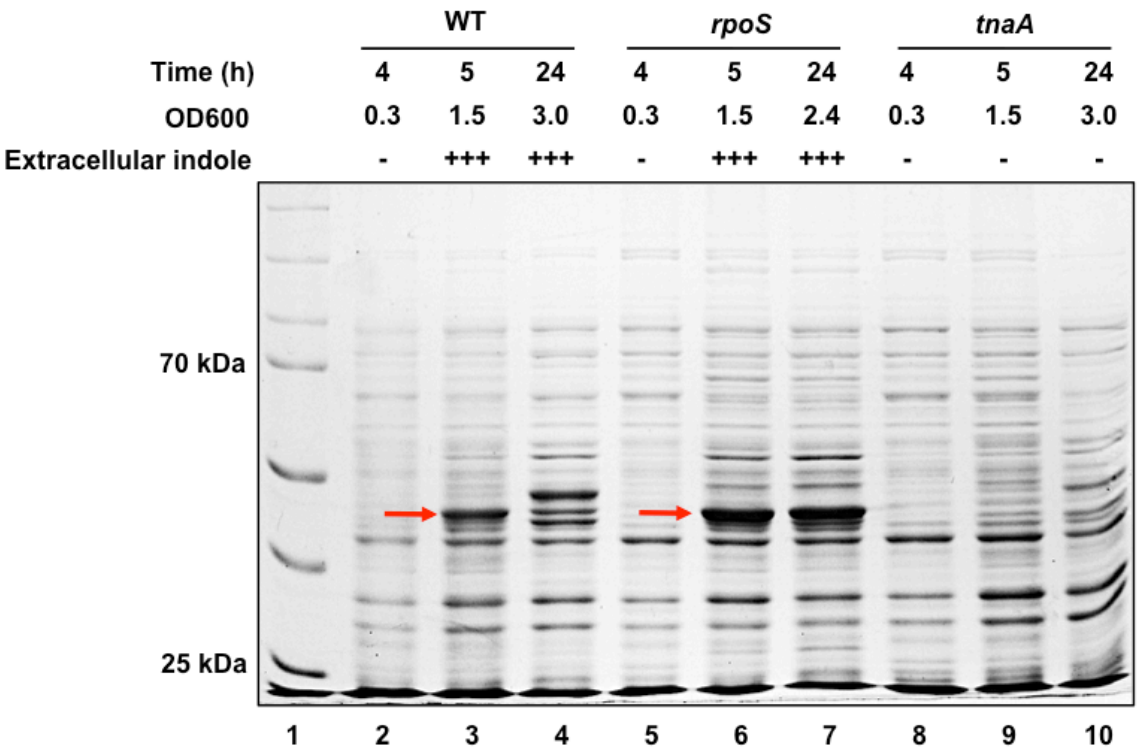

Figure 13: TnaA induction is stationary phase-specific and RpoS acts as a negative regulator of TnaA expression.

Overnight cultures of K12 MG1655, isogenic *rpoS* mutant, and the *tnaA* mutant grown in LB were diluted to 1:10000 in prewarmed LB and sampled at indicated OD600 values. Total cell proteins were resolved on an 8% polyacrylamide gel (the red arrow indicates the band containing the TnaA protein). Proteins were visualized after staining in 0.1% Coomassie Blue dye and subsequent destaining.

To further examine the role of RpoS in TnaA regulation, we tested TnaA levels in the isogenic *clpP* mutant. The ClpXP protease is responsible for low levels of RpoS in exponential phase. A *clpP* mutant exhibits elevated RpoS levels during exponential phase due to increased RpoS stability (160). Since RpoS reduces TnaA levels in long-term stationary phase, we predicted that the negative effect of RpoS on TnaA should be more pronounced in a *clpP* mutant. Indeed, TnaA was induced in the *clpP* mutant but the protein levels were lower compared to the WT cells (figure 14). In long-term stationary phase (i.e. 24 h – 48 h) of incubation, TnaA protein levels were substantially reduced compared to the WT strain. The amount of indole produced after 24 h of incubation was the same as the WT cells however suggesting that the amount of TnaA induced in the *clpP* mutant was sufficient to quantitatively convert the available tryptophan to indole (figure 14). Expression of RpoS itself was not affected by *tnaA* deletion (figure 14**)**. Intensity of a band identified to be completely dependent on RpoS for expression (labelled with green arrow in figure 14) was reduced in intensity in the *tnaA* mutant compared to WT. This band was identified to contain Gad/GadB (as mass spectrometry may not be able to distinguish these proteins). Indole acts synergistically with RpoS to control expression of proteins contained in this band (band labelled green in figure 14).

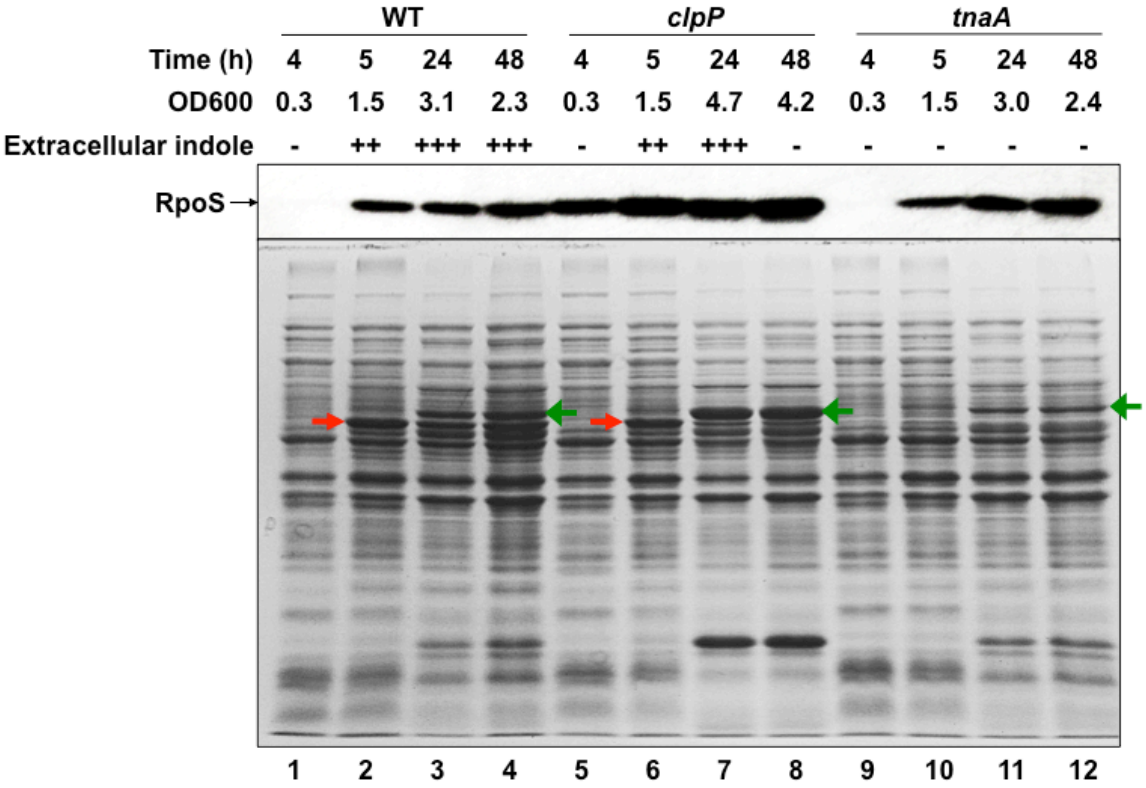

Figure 14: RpoS reduces TnaA levels in a dose-dependent manner.

Overnight cultures of *E. coli* K12 MG1655, isogenic *clpP* mutant, and the *tnaA* mutant grown in LB were diluted to 1:10000 in prewarmed LB and sampled at indicated OD600 values. Total cell proteins were resolved on an 8% polyacrylamide gel (the red arrow indicates the band containing the TnaA protein). RpoS was detected using immunoblots analysis while TnaA levels were deciphered from the protein gel stained with Coomassie Blue. The band indicated in green is completely dependent on RpoS for expression and was also reduced in intensity in the *tnaA* mutant. Using MALDI-TOF-MS, the band was identified as a Gad protein (Glutamate decarboxylase A/B).

It is well established that cAMP-CRP represses *rpoS* (24) but activates *tnaA* (168), so we tested the hypothesis that cAMP-CRP mediates its effects indirectly through RpoS during growth in glucose. As shown in figure 15, addition of glucose to LB repressed TnaA induction in both the WT and isogenic *rpoS* mutant strains suggesting that cAMP-CRP mediated catabolite repression of *tnaA* expression occurs independently of RpoS with glucose as a sole carbon source.

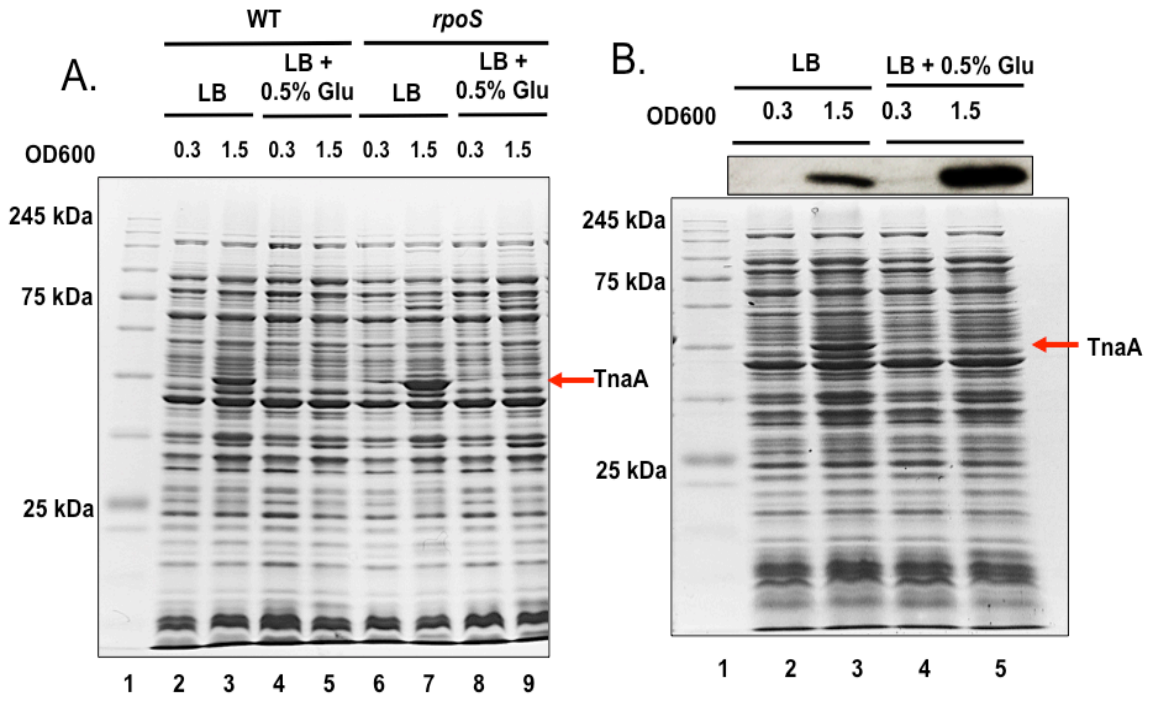

Figure 15: cAMP-CRP dependent catabolite repression of TnaA is independent of RpoS.

Overnight cultures of *E. coli* K12 MG1655 and the isogenic *rpoS* mutant, grown in LB, were diluted to 1:10000 in prewarmed LB or LB supplement with 0.5% glucose and sampled at indicated OD600 values. Total cell proteins were resolved on 12% polyacrylamide gel (the red arrow indicates the band containing the TnaA protein). B) During growth in glucose, RpoS levels are higher while induction of TnaA is not observed. A) During growth in glucose, *tnaA* induction is not observed during entry into stationary phase in both WT K12 MG1655 and isogenic *rpoS* strains. RpoS was detected using immunoblot analysis in B) while TnaA levels were deciphered from the protein gel stained with Coomassie Blue dye.

Regulation and identity of tryptophanase as made by observing band intensity from protein gels was also verified using a polyclonal anti-TnaA antibody (figure 16).

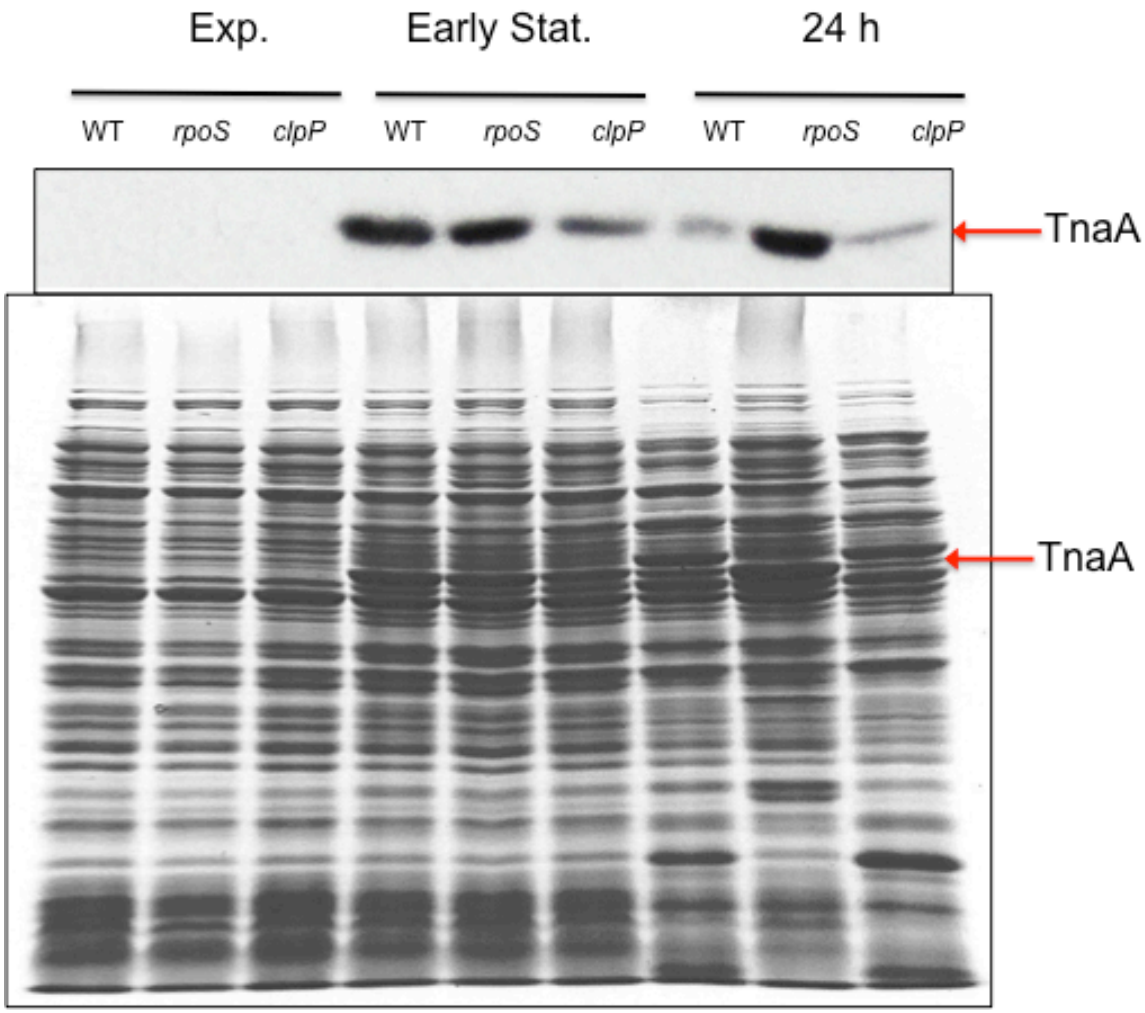

Figure 16: Verification of TnaA regulation with immunoblot analysis using a polyclonal anti-TnaA anti-body.

3.1.2 Expression dynamics of TnaA in O157:H7 compared to K12

RpoS controls a large set of genes in *E. coli* O157:H7 during entry into stationary phase (9). Since we have previously found an opposite effect of *rpoS* deletion on *tnaA* mRNA levels during early stationary phase in O157:H7 and the K12 laboratory strain (9, 11), we also examined TnaA protein expression dynamics and indole production in O157:H7 substrain EDL933. In comparison to the K12 MG1655 laboratory strain in which indole production began during late-exponential (OD600 = 0.7), O157:H7 and other environmental isolates (see below) produced indole much later (OD600 = 2.0) and relative levels of tryptophanase were much lower. The increased production of indole was concomitant with induction of tryptophanase. Like K12 MG1655, expression of TnaA was transient in O157:H7 as a result of RpoS-mediated negative regulation (expression of TnaA in O157:H7 Δ*rpoS* was consistently high during prolonged incubation, figure 17)).

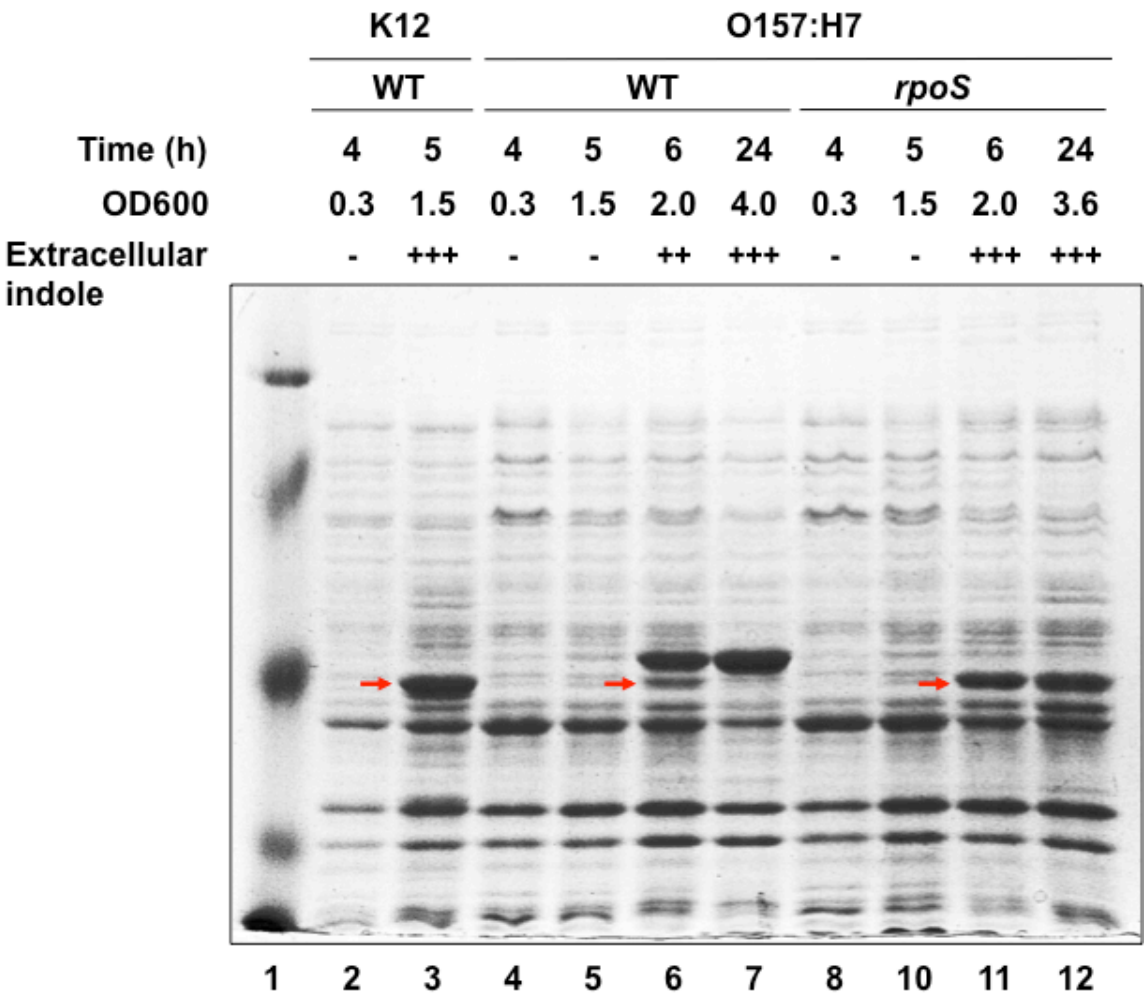

Figure 17: Effect of RpoS deletion on TnaA levels in O157:H7.

Overnight cultures of O157:H7 and isogenic *rpoS* mutant, grown in LB, were diluted to 1:10000 in prewarmed LB. Total cell proteins were resolved on an 8% polyacrylamide gel (the red arrow indicates the band containing the TnaA protein). Tryptophanase levels were deciphered from the protein gel stained with Coomassie Blue.

3.1.3 Growth phase-dependent switch in RpoS regulation of *tnaA*

Positive regulation in exponential phase and negative regulation in stationary phase

We have previously observed opposite regulation of *tnaA* at the transcript level in K12 MG1655 (positive regulation in exponential phase and negative regulation in stationary phase (11, 45)). The effect of *rpoS* deletion on relative transcript levels of *tnaA* between the WT and *rpoS* strains was tested in both exponential (OD600 = 0.3) and early stationary phases (OD600 = 1.5). *tnaA* was upregulated by about 3.0-fold upon *rpoS* deletion in early stationary phase and downregulated by about 4.0-fold during exponential phase (figure 18). *tnaA* was induced upon entry into stationary phase in both strains. The physiological significance of this opposite regulation is not entirely clear. *katE* (encoding an RpoS-controlled catalase) and *yliH* (a novel regulator of biofilm formation (16)) were used as control genes as *katE* is positively-controlled by RpoS while *yliH* is negatively-controlled during early stationary phase (11). As expected, *katE* expression was largely abolished upon *rpoS* deletion strain in early stationary phase while *yliH* was induced. These results suggest that the role of RpoS in regulating tryptophanase may be physiologically distinct depending on growth phase. The switch in transcriptional regulation occurs after the production of indole.

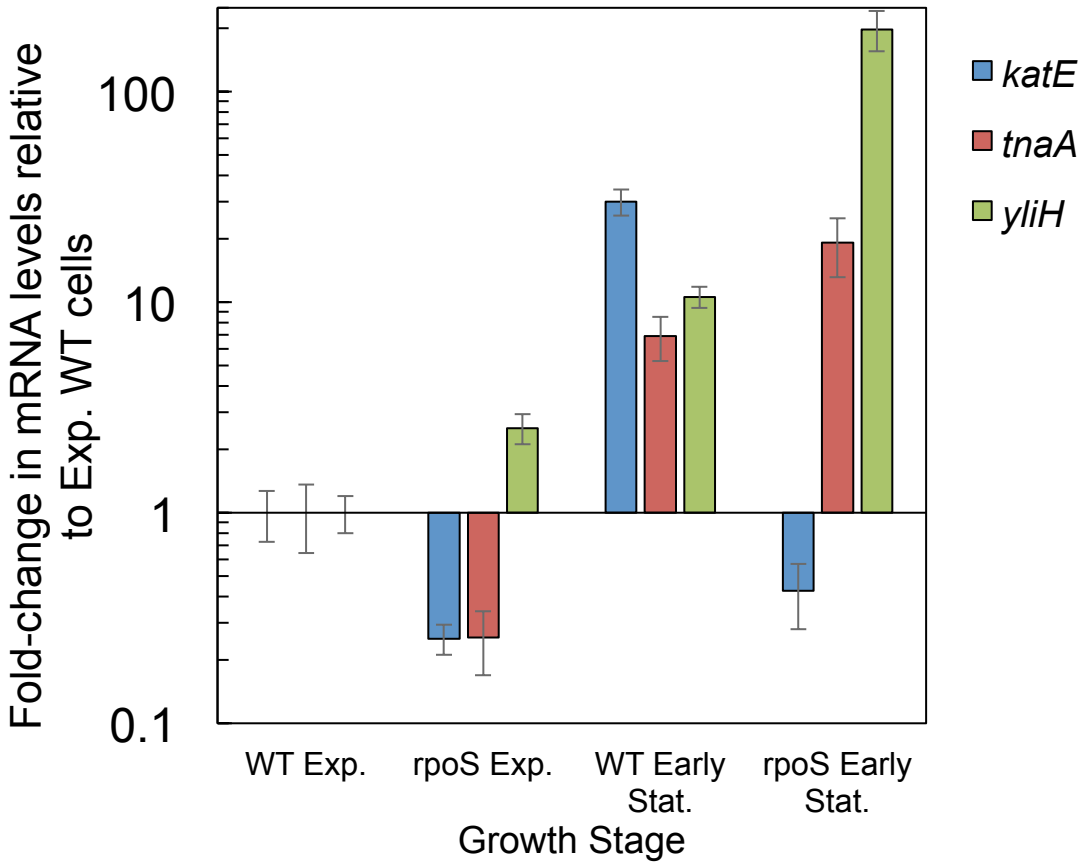

Figure 18: Relative *tnaA* mRNA levels in WT and isogenic *rpoS* mutant of *E. coli* K12 MG1655 during exponential phase (OD600 = 0.3) and early stationary phases (OD600 = 1.5).

Total RNA was isolated at indicated growth stages and reverse transcribed to cDNA. Relative abundance of the *tnaA, katE,* and *yliH* transcripts was quantified using 16S ribosomal RNA as a control gene.

3.1.4 Temporal dynamics of RpoS and indole signalling in environmental isolates of *E. coli* compared to K12 MG1655

Since both RpoS and indole play important physiological roles in *E. coli* and as expression of RpoS is highly variable in environmental strains of *E. coli* (167), we examined the dynamics of RpoS induction and indole production in selected environmental strains. RpoS levels in these strains were higher during exponential phase, peaked during early stationary phase (OD600 = 1.5), and declined after (figure 19). This contrasts with the K12 MG1655 laboratory strain, which expressed lower levels of RpoS in exponential phase, but more stably expressed RpoS during long-term stationary phase. Maximal indole production and tryptophanase expression occurred much later than RpoS induction in all the strains tested. It appears that RpoS induction takes place prior to indole signalling in all of the strains tested (figure 19).

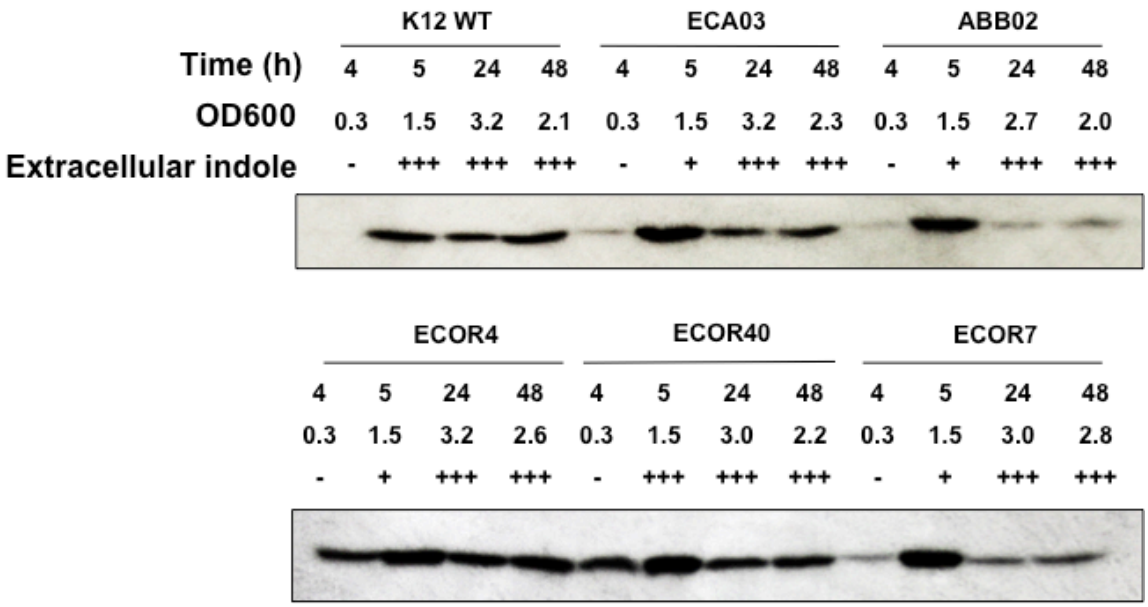

Figure 19: Growth phase-dependent levels of RpoS and extracellular indole in environmental isolates.

Overnight cultures of K12 MG1655 and selected environmental isolates, grown in LB, were diluted to 1:10000 in pre-warmed LB medium. Total cell protein was resolved on a 12% polyacrylamide gel and immunoblotted for RpoS using monoclonal anti-RpoS antibody. Total protein lysate was used as a loading control (not shown).

3.2 Discussion: Regulation of tryptophanase and indole signalling

Indole is a signalling molecule produced by Gram-positive and Gram-negative bacteria that alters gene expression in response to environmental signals (73). Indole production during entry into stationary phase occurs due to upregulation of tryptophanase which converts exogenous tryptophan into indole, pyruvate, and ammonia (166). During entry into stationary phase, a subset of genes is co-ordinately upregulated or downregulated under the control of the alternative sigma factor RpoS, ultimately resulting in adaptation to non-optimal growth conditions (11, 13, 41). At the transcript level, regulation of tryptophanase by RpoS is complex as RpoS acts as either a positive (9, 10, 45) or a negative regulator (11) which led us to examine the role of RpoS in tryptophanase regulation at the protein and transcript level and extend our findings to environmental isolates. Our results indicate that RpoS is not necessary for full induction of tryptophanase during entry into stationary phase suggesting that the primary activating mechanism may require other regulators such as tryptophan and cAMP-CRP (165, 168). Our findings indicate that RpoS exerts a negative effect on tryptophanase however, causing a transient induction during prolonged growth in rich media. Hence, RpoS may serve to limit metabolic burden associated with TnaA, a highly expressed protein, after exogenous tryptophan has been converted to indole. *rpoS* deletion leads to a higher and sustained expression of tryptophanase during long-term growth. This may have physiological implications for the *rpoS* mutant cells resulting from altered indole signalling, however, this hypothesis was not tested. We also found that the final extracellular indole concentrations in the WT and *rpoS* cells are similar and as such, the

higher tryptophanase levels in the *rpoS* background may not have a physiological effect on endogenous processes. This supports the observations that the amount of indole produced is directly proportional to the amount of exogenous tryptophan and that *E. coli* maintains its internal pool of tryptophan during growth (76). However, it has recently been shown that the high transient intracellular indole levels rather than the extracellular levels play a major role in long term cell viability and growth (75). Therefore, it would be interesting to accurately measure intracellular indole levels in the WT and the isogenic *rpoS* cells to test the hypothesis that the intracellular indole levels are higher within the *rpoS* cells. Furthermore, we found that the negative regulation of TnaA by RpoS also occurs in the pathogenic O157:H7. This also results in a transient induction of tryptophanase in O157:H7. However, indole signalling (the induction of tryptophanase and generation of indole) occurs much later in O157:H7 (OD600 ~ 2.0). This was corroborated in environmental isolates of *E. coli,* which produce indole around the same time during growth in rich medium. The higher TnaA levels and early induction of TnaA and indole signalling seen in K12 MG1655 compared to environmental isolates may be a physiological adaptation as a result of years of culturing in the laboratory. A model of tryptophanase regulation is summarized in figure 20.

We confirm our previous findings of opposite regulation of *tnaA* by RpoS in exponential compared to stationary phase and additionally show that RpoS acts as a positive regulator of *tnaA* during exponential phase and a negative regulator during stationary phase, after production of indole. The mechanism or physiological significance of this switch in regulation is not clear, however, it may suggest an additional layer of

control in regulating TnaA and indole signalling that *E. coli* may use in diverse environments. Negative regulation by RpoS (11) can occur through sigma factor competition with the vegetative sigma factor RpoD for promoter binding (169) or by direct mechanisms such as DNA-binding to a promoter causing occlusion of other sigma factors at the promoter as reported in *Salmonella* (170).

The regulatory interaction of RpoS and tryptophanase has important implications in the field of biofilm formation. Both RpoS and tryptophanase have been found as positive and negative regulators of biofilm formation. After 48 h of incubation in rich medium, the isogenic *rpoS* mutants formed more biofilm than the WT strain. Biofilm formation in the isogenic *rpoS* strain also occurred earlier than the WT strain suggesting negative regulation by RpoS (171). In chemostat cultures however, RpoS has a positive effect on biofilm formation (172). Similarly, indole is proposed to be both a positive (15, 81) and a negative (16, 82) signal for biofilm formation. Most studies on biofilm formation have explored the role of either RpoS or indole signalling however since RpoS levels affect tryptophanase levels, this interaction is likely important in the process of biofilm formation. For example, variation in RpoS levels in different cells within a biofilm may cause heterogeneity in intracellular indole levels.

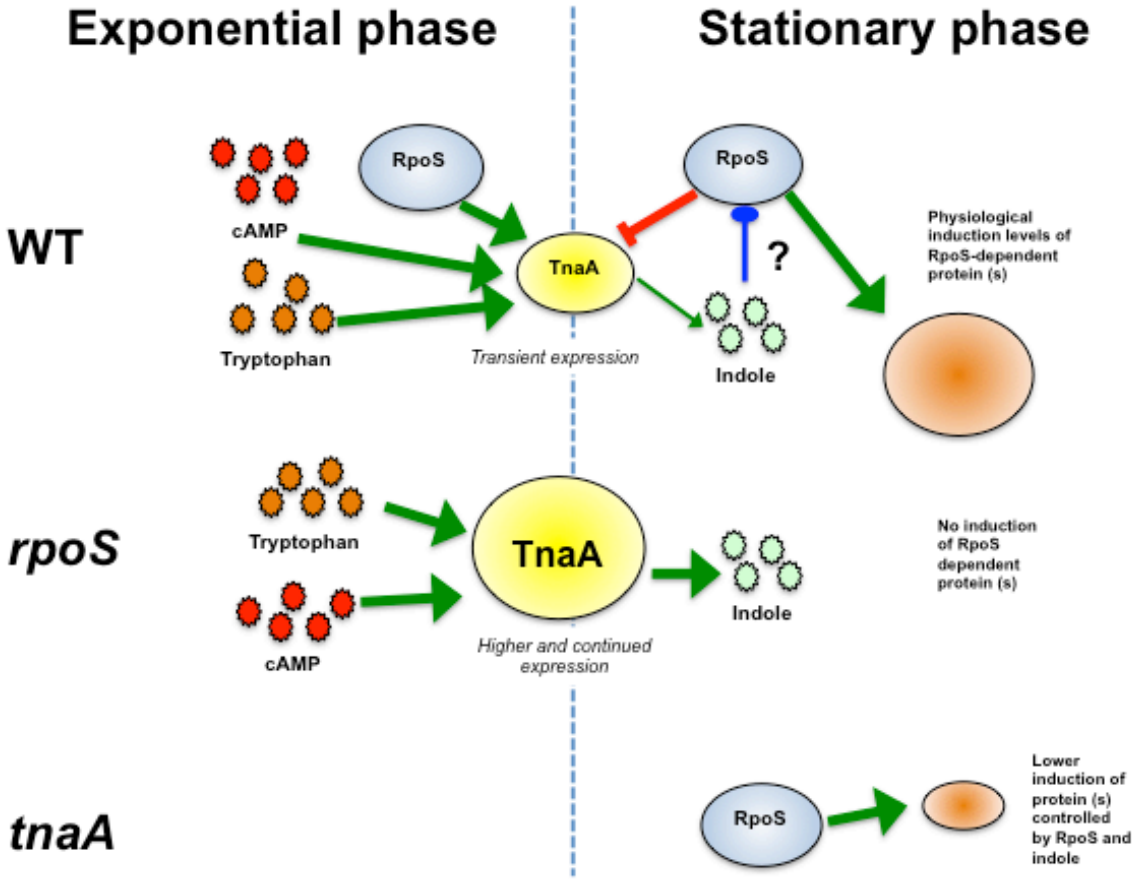

Figure 20: Regulation of tryptophanase during physiological growth stages in rich medium in WT and isogenic *rpoS* cells.

tnaA is induced during entry into stationary phase in both WT and *rpoS* strains however, expression is higher in the mutant cells. During stationary phase in WT cells, RpoS serves to limit the expression of tryptophanase following initial induction whereas TnaA levels remain high in the *rpoS* mutant. RpoS-mediated stationary phase adaptation occurs prior to induction of tryptophanase and indole production. Levels of at least one protein, completely dependent on RpoS for induction, are dependent additively on indole (this protein was identified as GadA/B using band excision and MALDI-TOF-MS).

Appendix 1: RNA integrity and quality check (RNA samples used for RT-qPCR experiments in Chapter 3)

Table 2: Yield and quality of RNA isolated from exponential phase (OD600 = 0.3) and early stationary phase (OD600 = 1.5) cells from replicate 1.

	Concentration (µg/µl)	Total volume (µl)	Yield (µl)	Abs 260/280
WT OD600 = 0.3	1.7	50	85	2.1
rpoS OD600 = 0.3	1.5	50	75	2.1
WT OD600 = 1.5	1.1	50	55	2.1
rpoS OD600 = 1.5	1.0	50	50	2.1

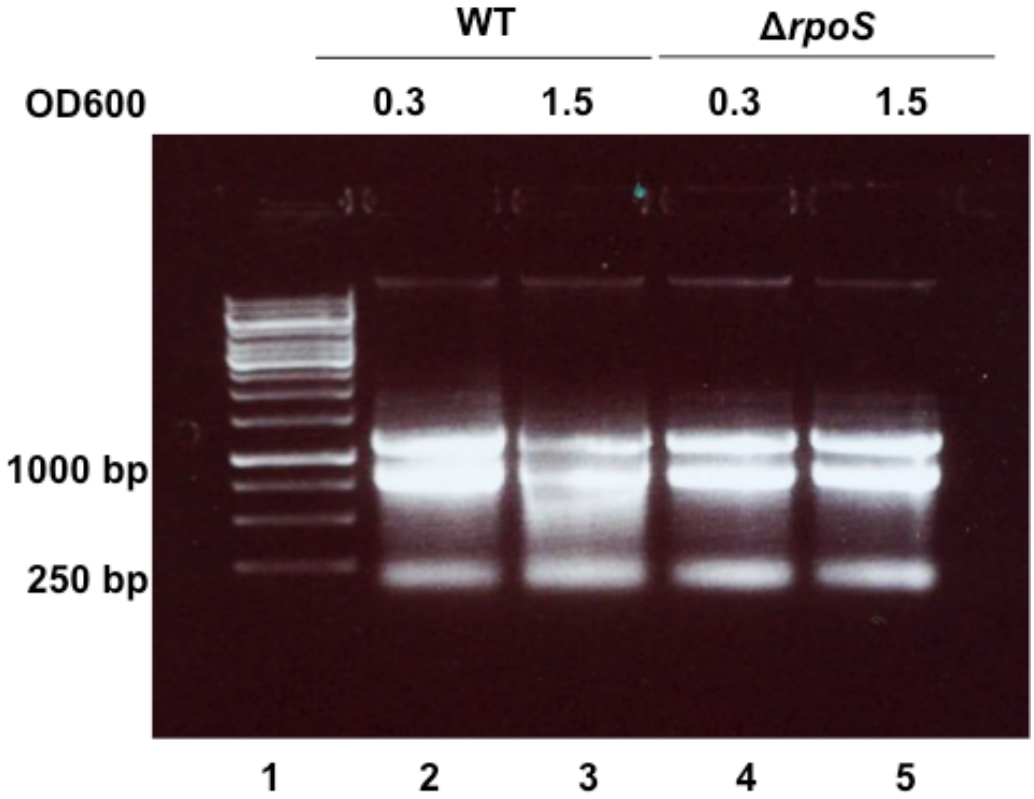

Figure 21: RNA integrity check for biological replicate 1 samples.

A 3-µl aliquot of RNA was run on a 0.8% agarose gel to check for integrity (gel for replicate 1).

Table 3: Yield and quality of RNA isolated from exponential phase (OD600 = 0.3) and early stationary phase (OD600 = 1.5) cells from replicate 2.

	Concentration (μg/μl)	Total volume (μl)	Yield (μg)	Abs 260/280
WT OD600 = 0.3	1.8	50	90	2.1
rpoS OD600 = 0.3	1.9	50	95	2.0
WT OD600 = 1.5	0.9	50	45	1.9
rpoS OD600 = 1.5	1.5	50	75	2.1

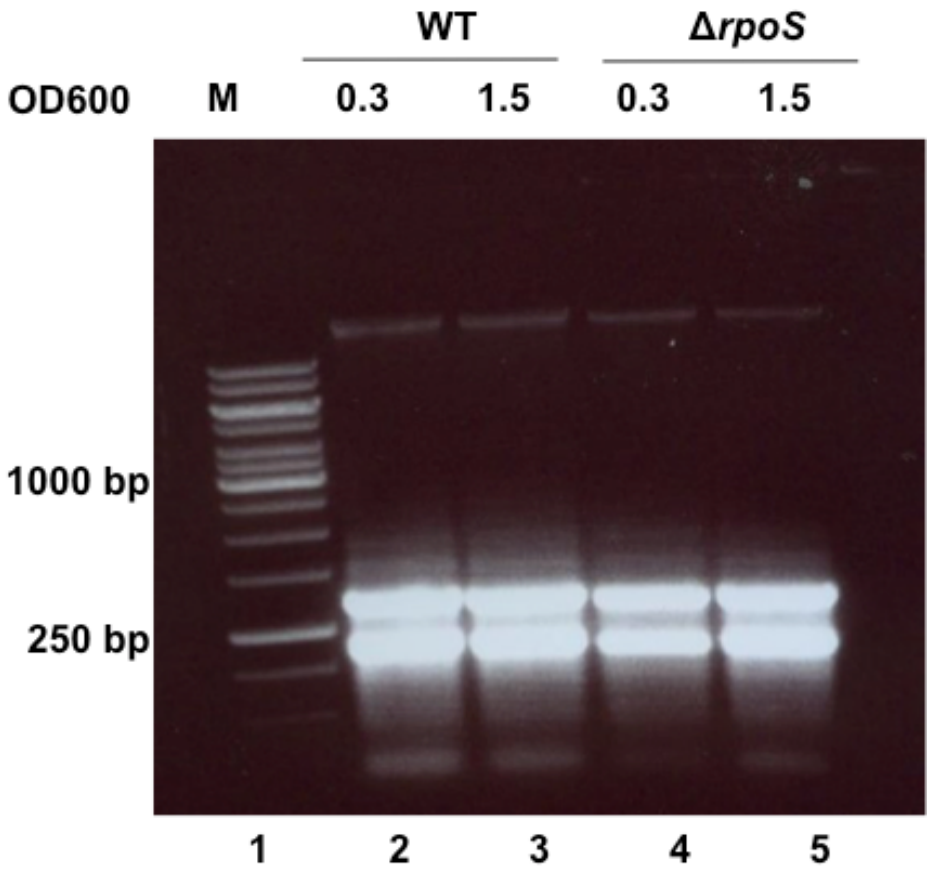

Figure 22: RNA integrity check for biological replicate 2 samples.

A 3-µl aliquot of RNA was run on a 0.8% agarose gel to check for integrity (gel for replicate 1).

Table 4: Yield and quality of RNA isolated from exponential phase (OD600 = 0.3) and early stationary phase (OD600 = 1.5) cells from replicate 3.

	Concentration (µg/µl)	Total volume (µl)	Yield (µg)	Abs 260/280
WT OD600 = 0.3	2.1	50	105	2.05
rpoS OD600 = 0.3	1.9	50	95	2.02
WT OD600 = 1.5	1.5	50	75	2.07
rpoS OD600 = 1.5	1.5	50	75	2.10

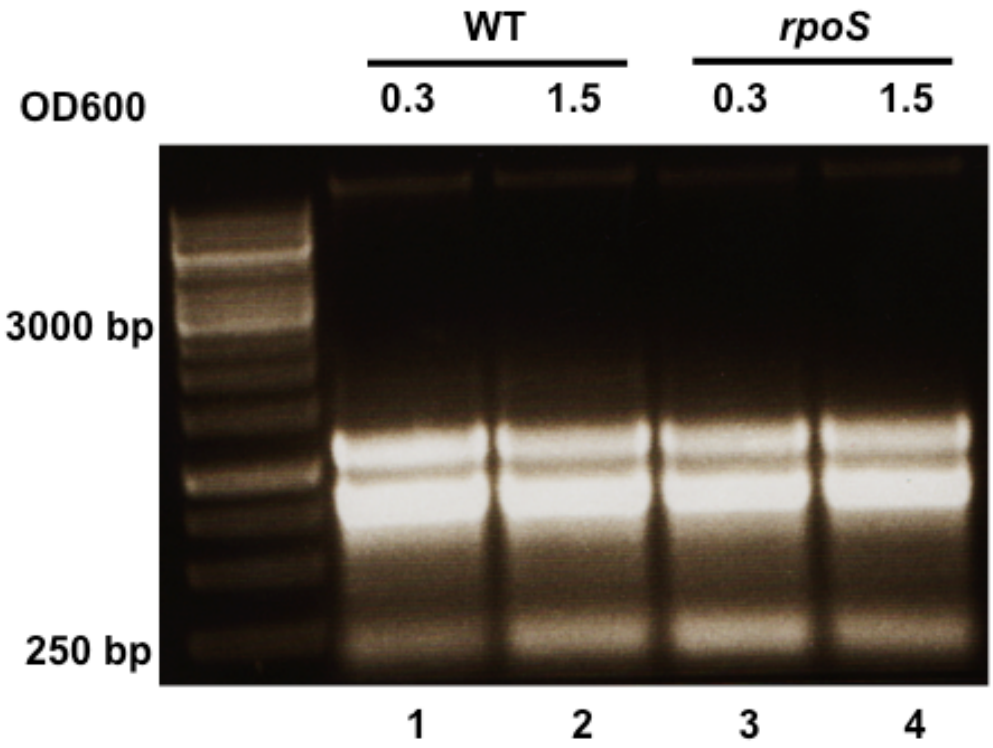

Figure 23: RNA integrity check for biological replicate 3 samples.

A 3-µl aliquot of RNA was run on a 0.8% agarose gel to check for integrity (gel for replicate 3).

Quality check after DNase 1 treatment of RNA

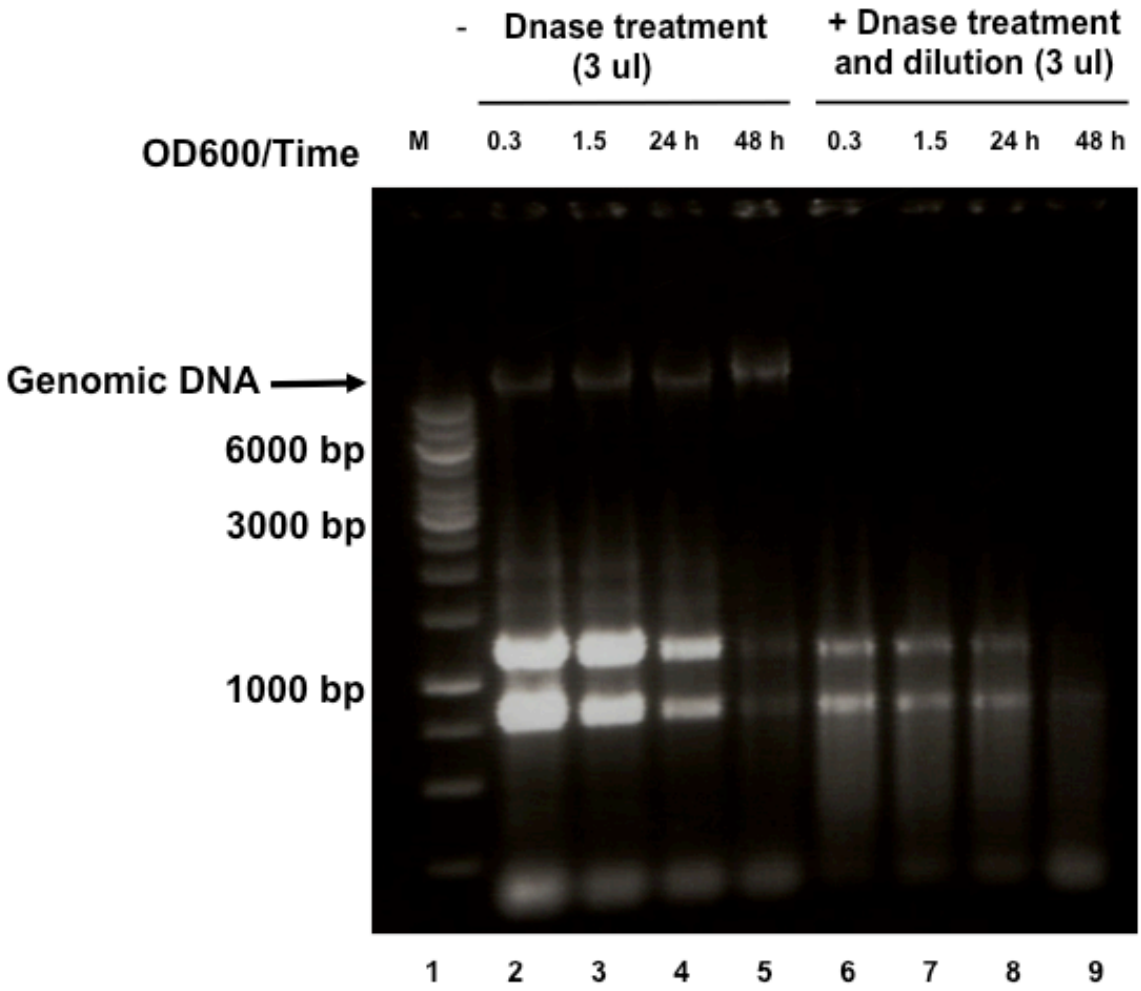

Figure 24: Quality check after in-solution DNase 1 treatment and re-purification of RNA.

Gene expression experiments can be severely compromised by contaminating genomic DNA in RNA preparations. In-solution DNase treatment was performed and RNA was repurified and diluted prior to quantification using RT-qPCR. Disappearance of the band of genomic DNA was verified and also no-reverse transcription control was run in all RT-qPCR experiments to check for genomic DNA contamination. RNA samples are from *E. coli* K12 MG1655.

Appendix 2: Gene deletions confirmations

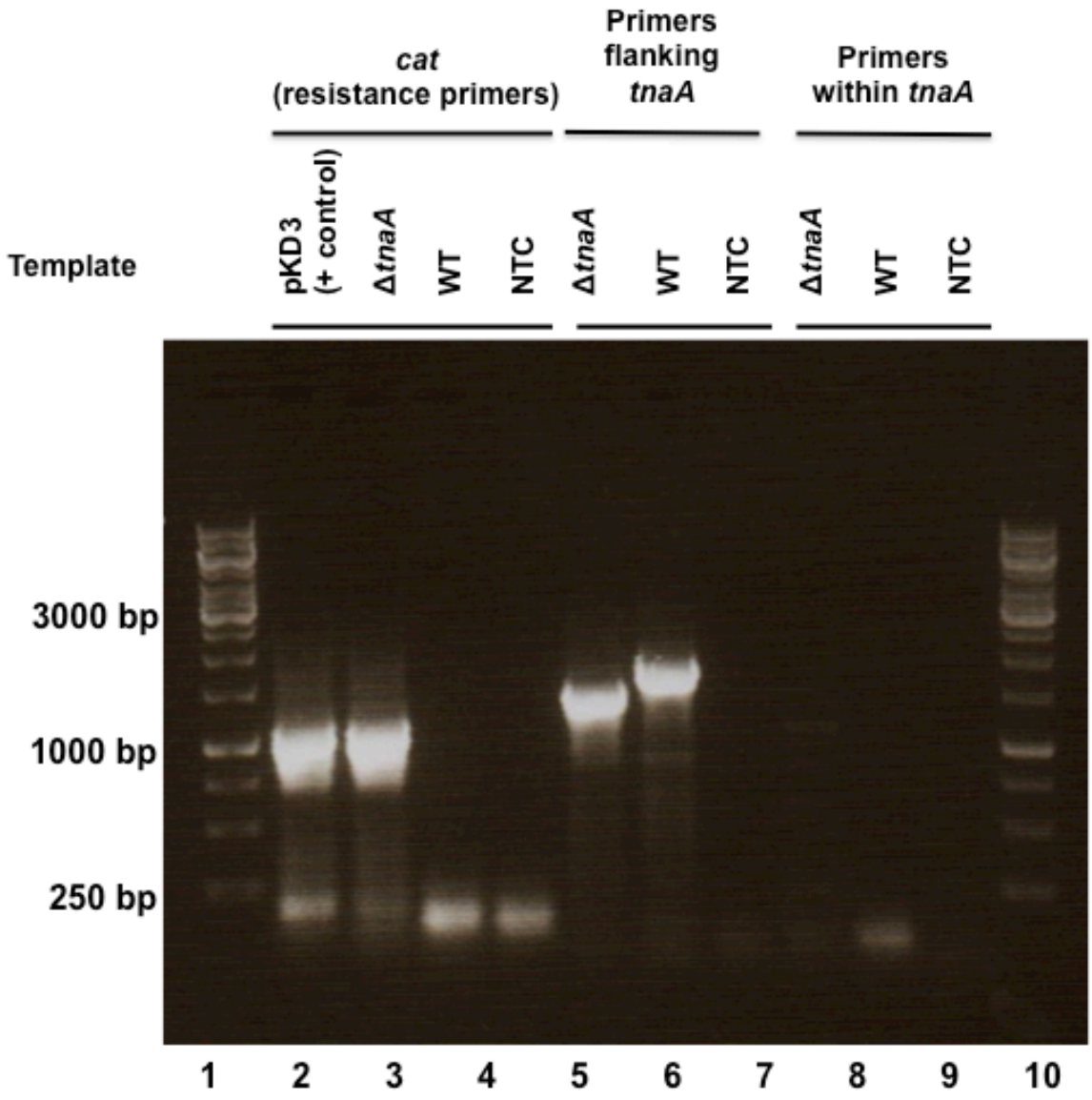

Figure 25: Confirmation PCR for *tnaA* deletion.

The chloramphenicol resistance cassette was amplified from the putative mutant (selected on chloramphenicol plates). The insertion of the cassette in the right location was verified using flanking primers. The absence of the *tnaA* gene was confirmed using primers that bind within the gene.

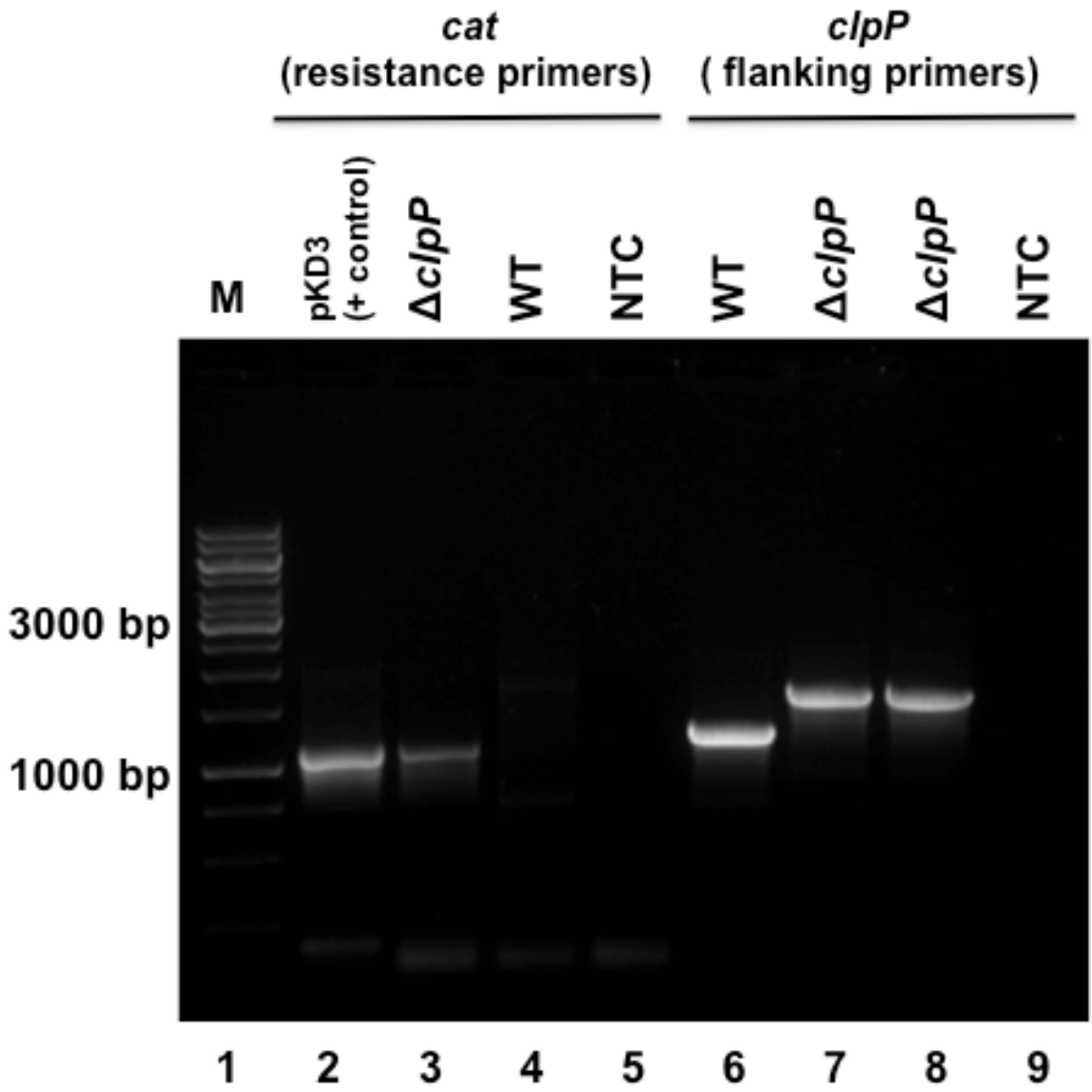

Figure 26: Confirmation PCR for *clpP* deletion.

The chloramphenicol resistance cassette was amplified from the putative mutant (selected on chloramphenicol plates). The insertion of the cassette in the right location was verified using flanking primers. The absence of the *tnaA* gene was confirmed using primers that bind within the gene.

Appendix 3: Real Time PCR validation experiments

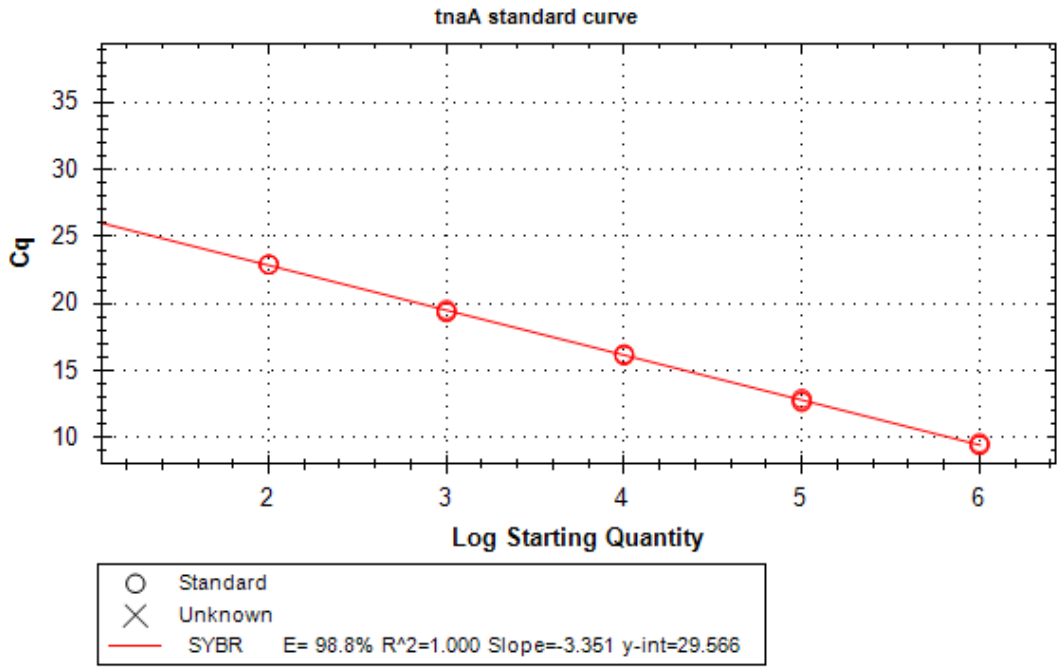

Figure 27: qPCR standard curve to test for amplification efficiency of the *tnaA* amplicon.

Standard curve using 10-fold serial dilution of *E. coli* K12 MG1655 genomic DNA to test for efficiency of amplification of the *tnaA* gene amplicon.

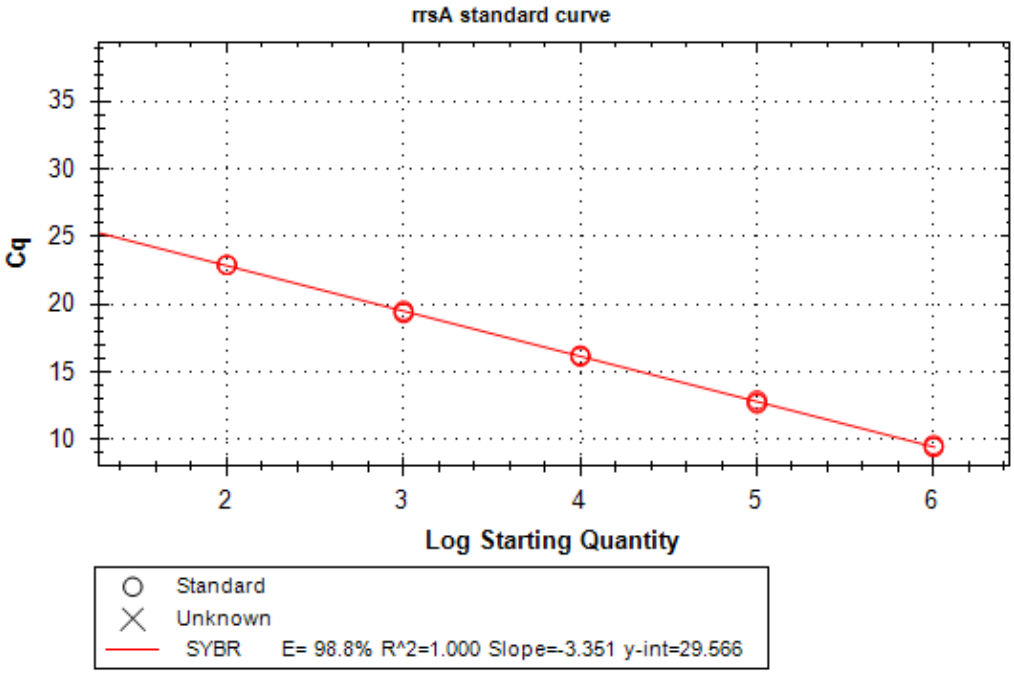

Figure 28: qPCR standard curve to test for amplification efficiency of the *rrsA* amplicon.

Standard curve using 10-fold serial dilution of *E. coli* K12 MG1655 genomic DNA to test for efficiency of amplification of the *rrsA* gene amplicon.

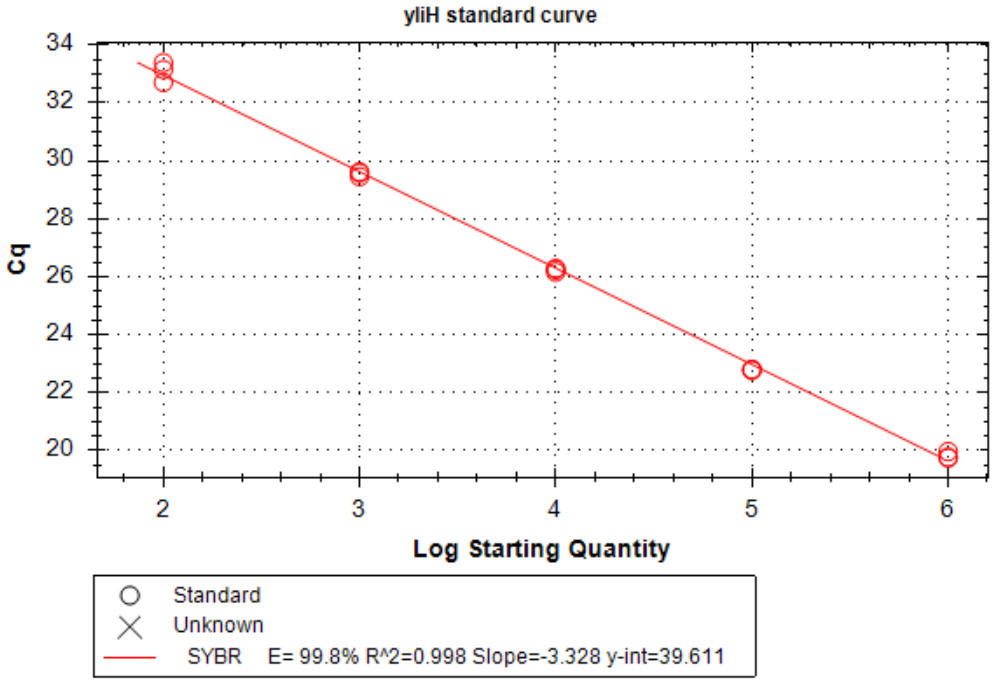

Figure 29: qPCR standard curve to test for amplification efficiency of the *yliH* amplicon.

Standard curve using 10-fold serial dilution of *E. coli* K12 MG1655 genomic DNA to test for efficiency of amplification of the *yliH* gene amplicon.

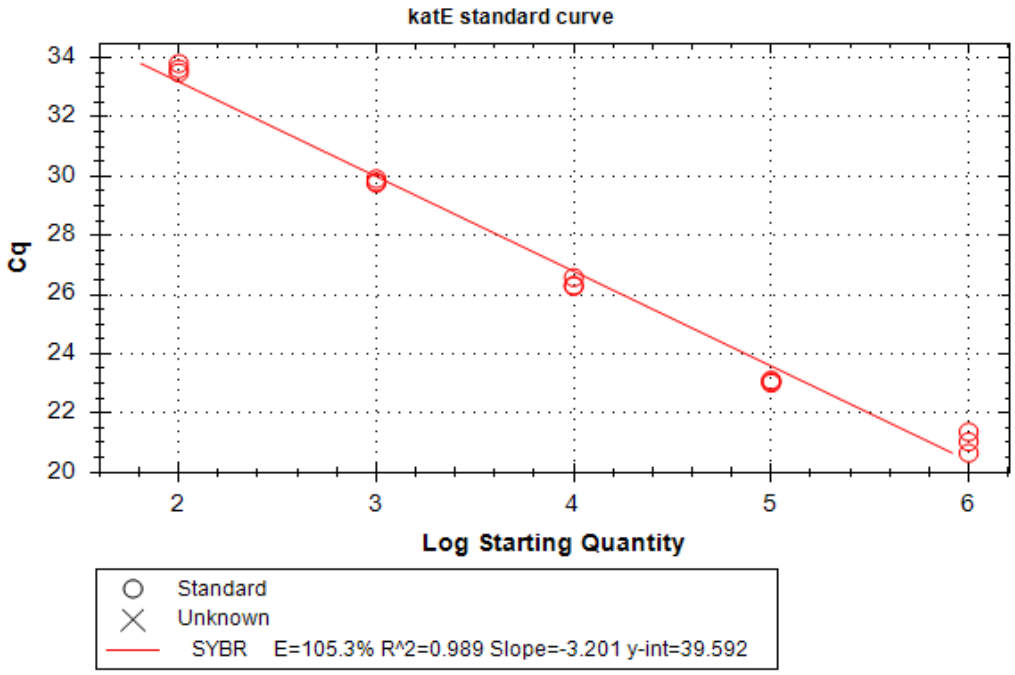

Figure 30: qPCR standard curve to test for amplification efficiency of the *katE* amplicon.

Standard curve using 10-fold serial dilution of K12 MG1655 genomic DNA to test for efficiency of amplification of the *katE* gene amplicon.

Melt curve checks for primer specificity

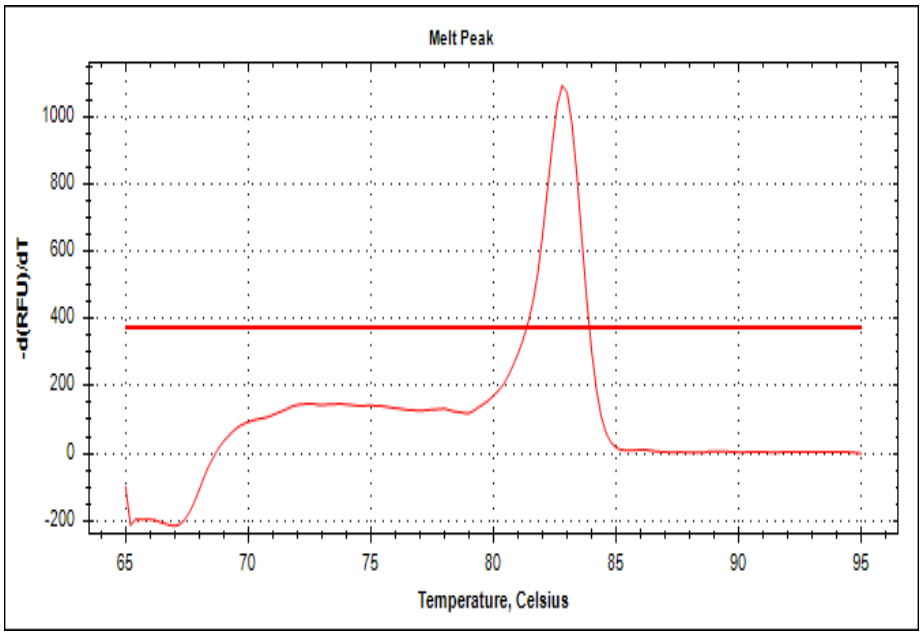

Figure 31: Melt curve analysis of the *tnaA* amplicon.

Single melt curve peak indicates that the amplification is specific and no non-specific products are observed.

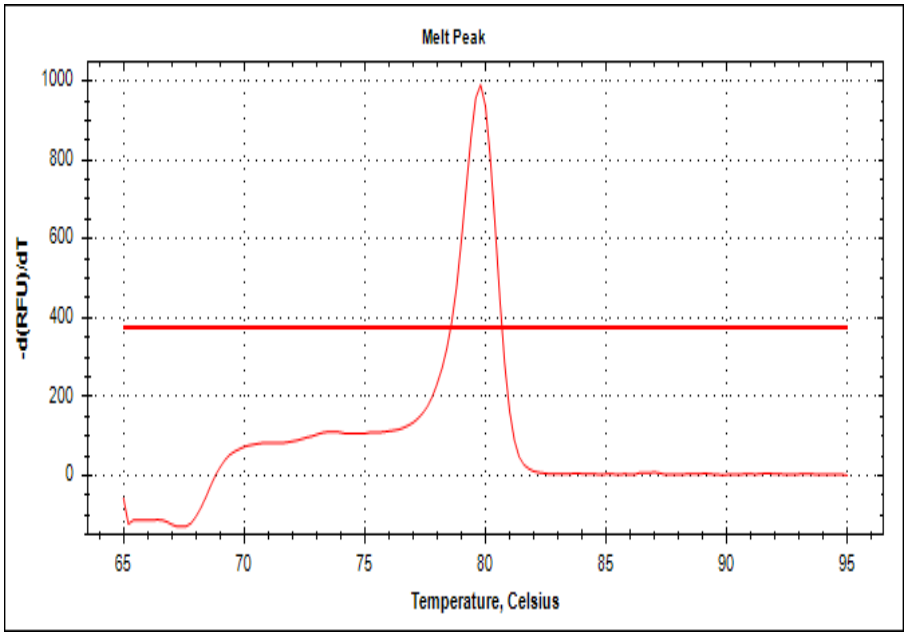

Figure 32: Melt curve analysis of the *yliH* amplicon.

Single melt curve peak indicates that the amplification is specific and no non-specific product is formed.

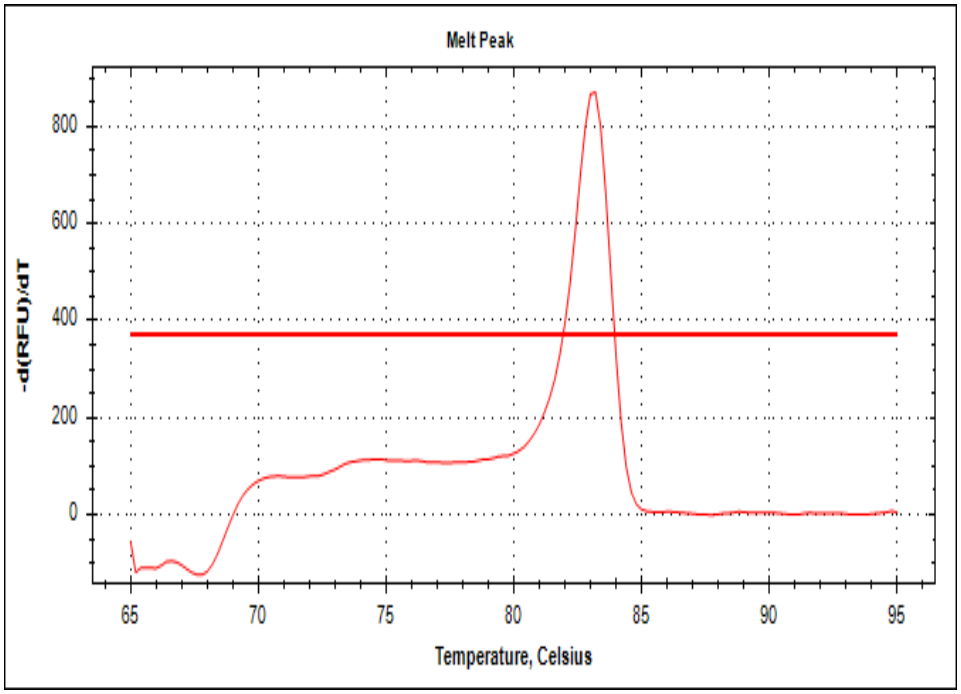

Figure 33: Melt curve analysis of the *katE* amplicon.

Single melt curve peak indicates that the amplification is specific and no non-specific product is formed.

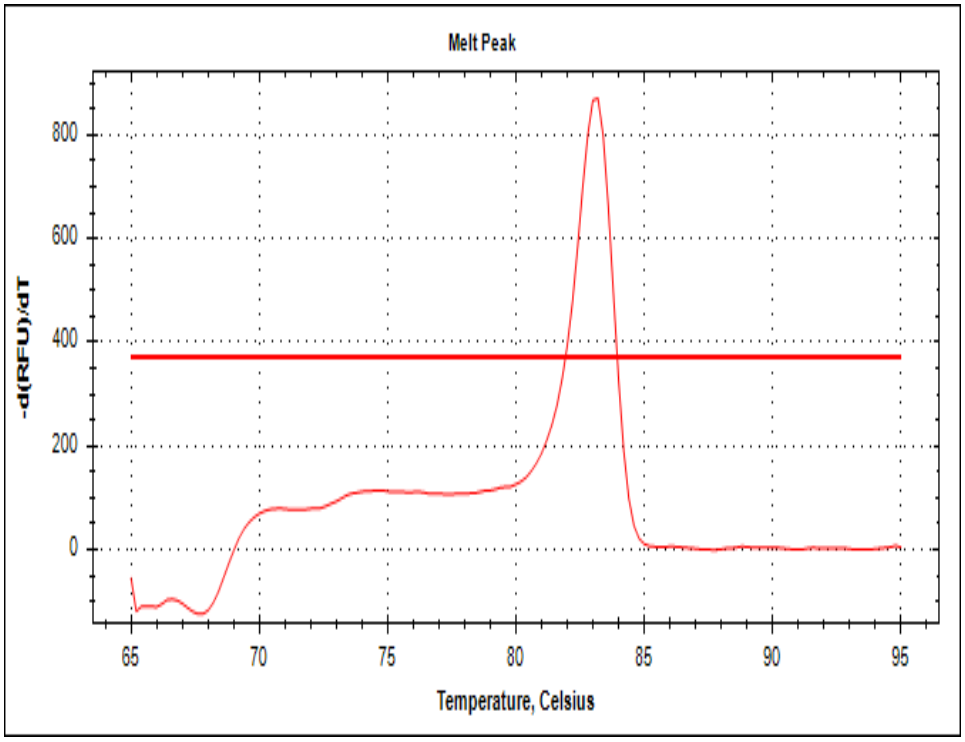

Figure 34: Melt curve analysis of the *rrsA* amplicon.

Single melt curve peak indicates that the amplification is specific and no non-specific product is formed.

Appendix 4: Peak lists for MALDI-TOF-MS identification of proteins

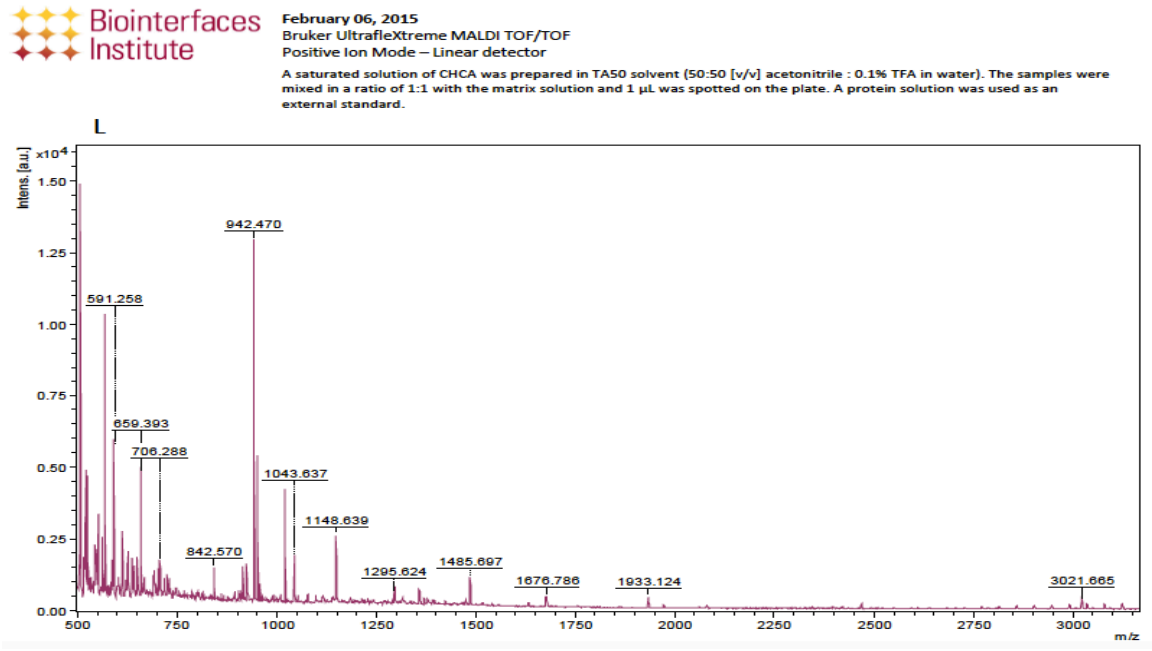

Figure 35: Peak list of mass to charge ratios of tryptic peptides obtained from the digestion of the Dps band with porcine trypsin.

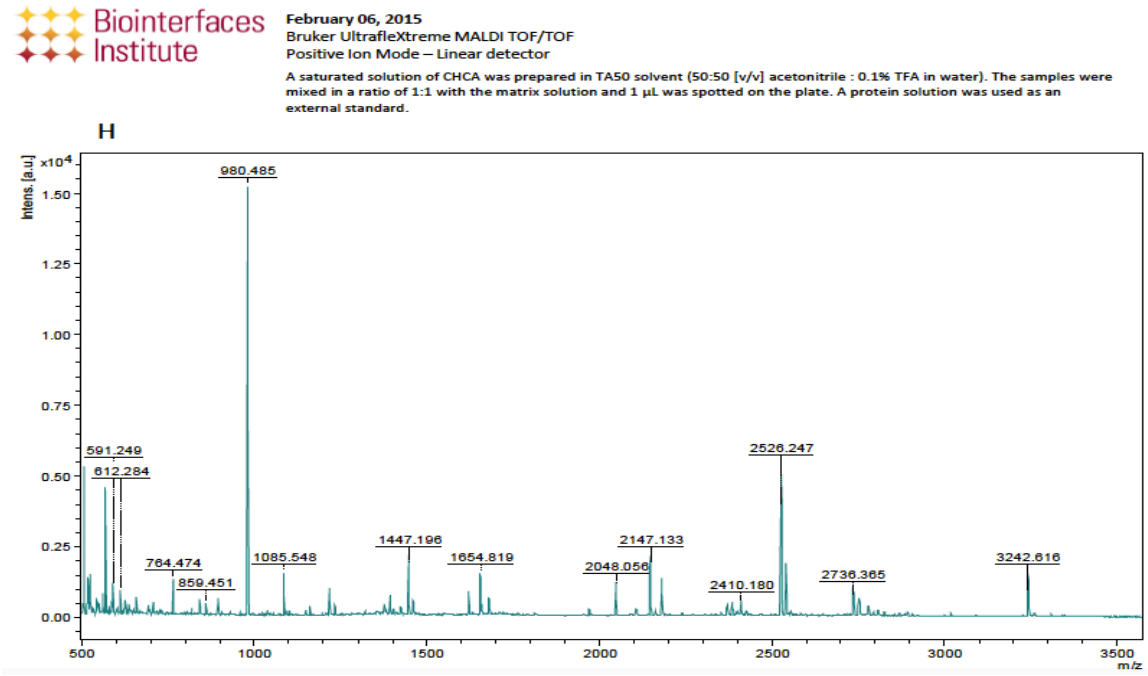

Figure 36: Peak list of mass to charge ratios of tryptic peptides obtained from the digestion of the GadA/B band with porcine trypsin.

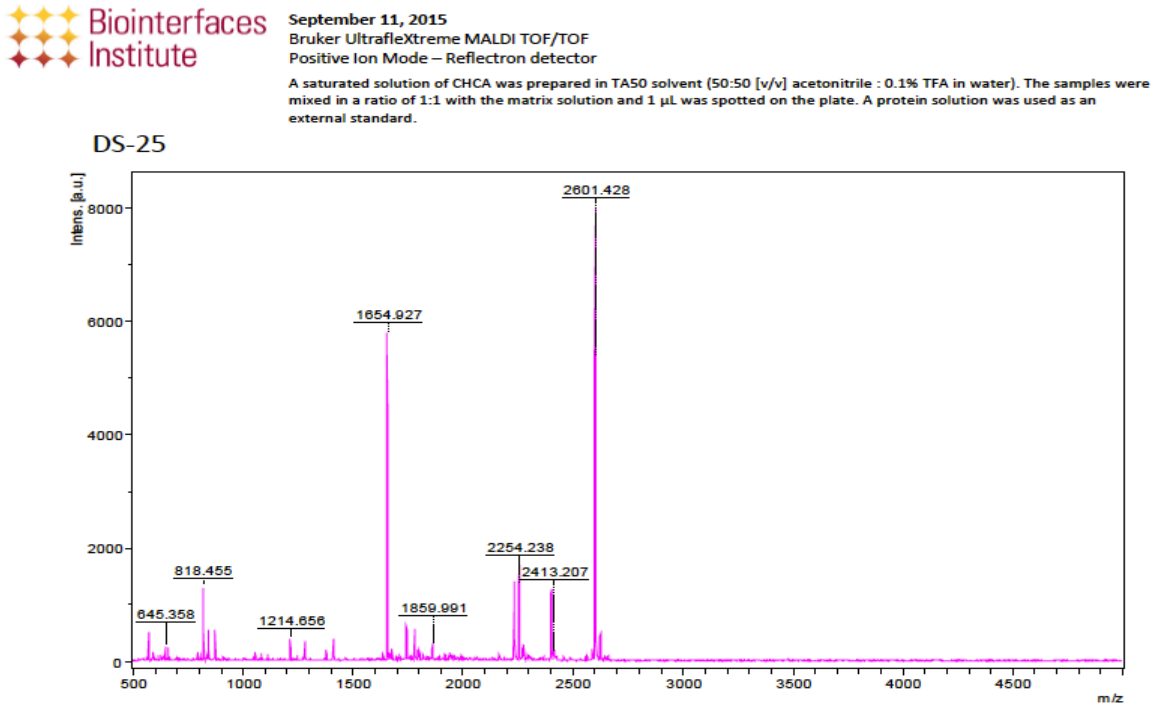

Figure 37: Peak list of mass to charge ratios of tryptic peptides obtained from the digestion of the OmpA band with porcine trypsin.

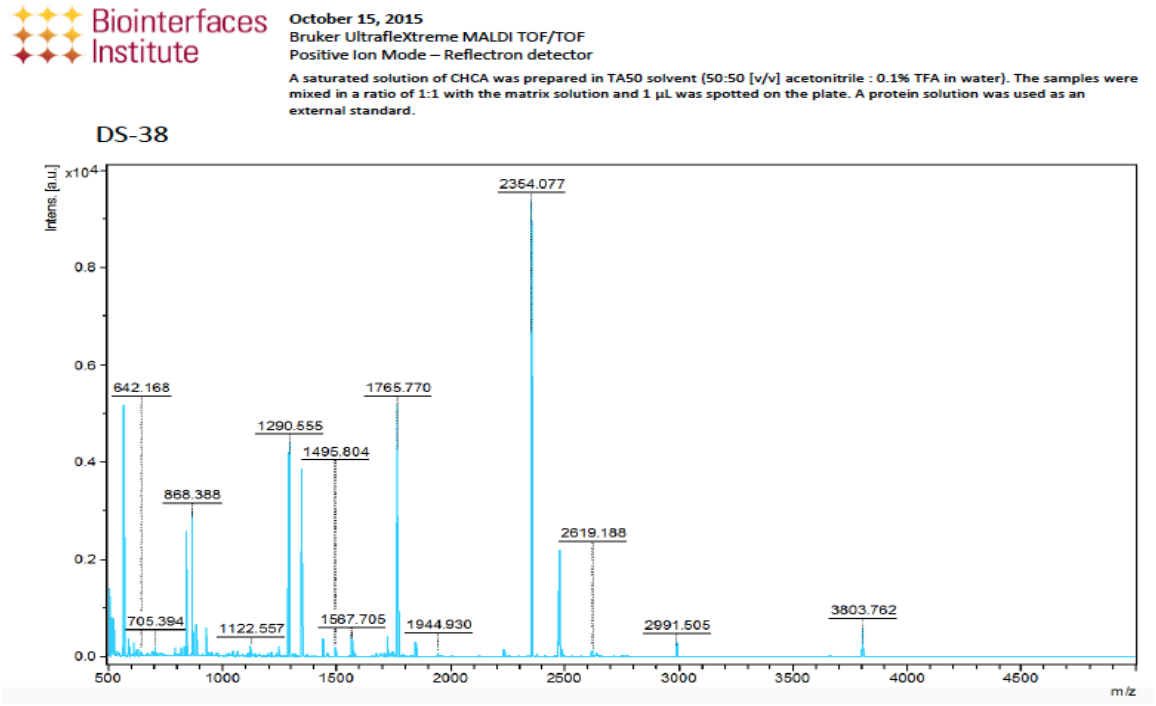

Figure 38: Peak list of mass to charge ratios of tryptic peptides obtained from the digestion of the OmpC band with porcine trypsin.

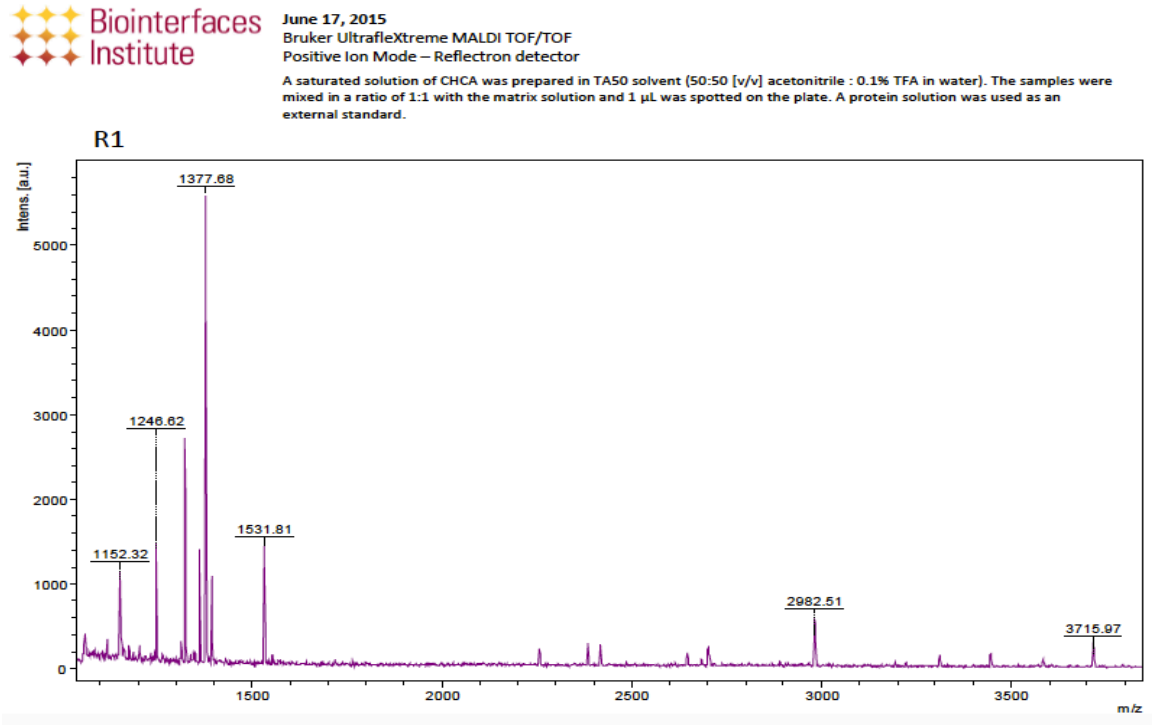

Figure 39: Peak list of mass to charge ratios of tryptic peptides obtained from the digestion of the OmpW band with porcine trypsin.

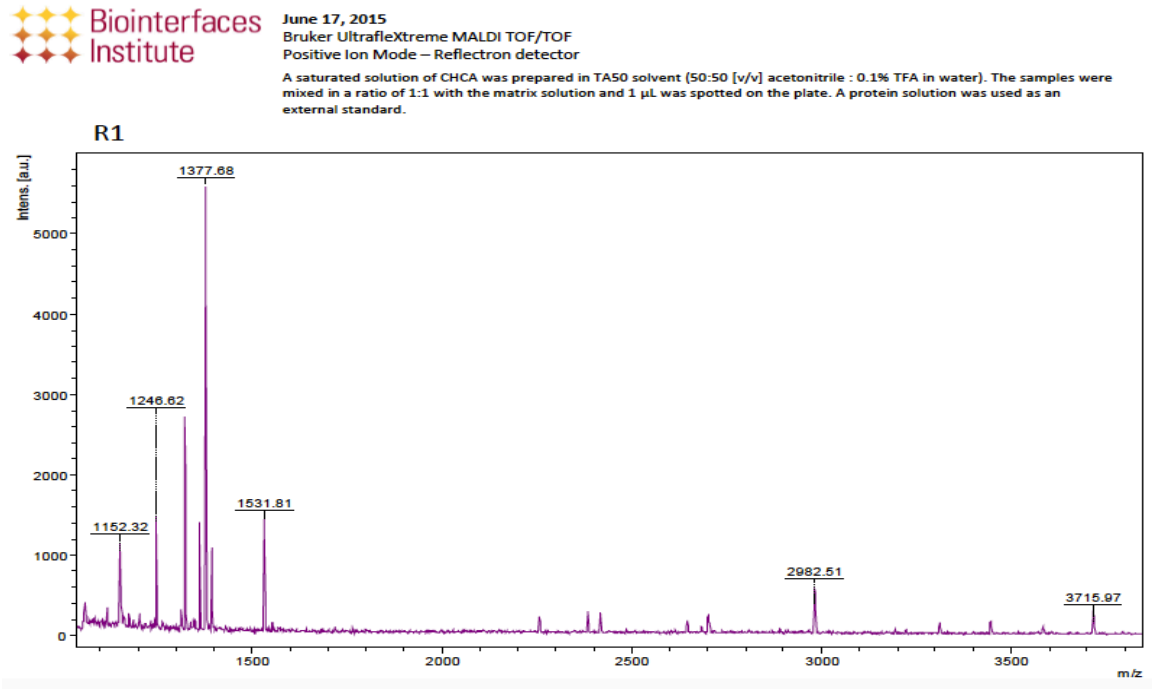

Figure 40: Peak list of mass to charge ratios of tryptic peptides obtained from the digestion of the TufA band with porcine trypsin.

Appendix 5: Raw data for RT-qPCR

Table 5: Raw Cq values corresponding to data presented in figure 18

Growth stage	Gene and Sample	Rep 1	Rep 2	Rep 3
Exp. Phase (OD600 = 0.3)	WT *tnaA*	23.17, 22.85	22.21, 22.07	21.07, 20.73
Exp. Phase (OD600 = 0.3)	WT *katE*	30.58, 30.51	31.25, 30.95	30.57, 30.70
Exp. Phase (OD600 = 0.3)	WT *yliH*	34.19, 33.67	33.25, 32.90	33.90, 33.87
Exp. Phase (OD600 = 0.3)	WT *rrsA*	11.57, 11.68	11.04, 10.43	11.29, 10.71
Exp. Phase (OD600 = 0.3)	*rpoS tnaA*	23.18, 23.10	25.43, 25.32	24.29, 24.33
Exp. Phase (OD600 = 0.3)	*rpoS katE*	32.65, 33.43	32.50, 32.69	34.08, 33.42
Exp. Phase (OD600 = 0.3)	*rpoS yliH*	33.60,32.72	31.95, 31.54	32.62, 33.30
Exp. Phase (OD600 = 0.3)	*rpoS rrsA*	11.65, 11.73	10.17,11.05	12.16, 12.19
Early. Stat. Phase (OD600 = 1.5)	WT *tnaA*	19.92,19.79	18.17, 17.97	19.80, 19.66
Early. Stat. Phase (OD600 = 1.5)	WT *katE*	25.70, 25.81	26.36, 26.40	26.09, 26.04
Early. Stat. Phase (OD600 = 1.5)	WT *yliH*	30.55, 30.35	29.57, 29.46	30.40, 30.91
Early. Stat. Phase (OD600 = 1.5)	WT *rrsA*	10.76, 10.86	10.83, 11.30	11.43, 11.94
Early. Stat. Phase (OD600 = 1.5)	*rpoS tnaA*	17.12, 16.84	16.44, 16.30	18.59, 18.32
Early. Stat. Phase (OD600 = 1.5)	*rpoS katE*	30.73, 30.88	30.73, 30.94	33.19, 33.67
Early. Stat. Phase (OD600 = 1.5)	*rpoS yliH*	25.51, 25.44	25.22, 25.09	25.97, 26.07
Early. Stat. Phase (OD600 = 1.5)	*rpoS rrsA*	10.09, 10.20	9.86, 9.88	12.74, 11.71

Cq values for gene expression analysis of *tnaA*, *katE*, and *yliH* in *E. coli* K12 MG1655 in exponential (OD600 = 0.3) and early stationary (OD600 = 1.5) phases of growth. Technical replicates are separated by a comma. No template control (NTC) and No reverse transcription control (NRT) were run for each reaction to check for genomic DNA contamination of RNA samples.

Appendix 6: List and RFU data of genes active during long-term incubation

Table 6: List and RFU (background subtracted and normalized to OD600) data of genes active during long-term incubation.

Gene	Function	6 h	24 h	48 h	120 h	168 h	Notes from *Ecocyc*
hisL	Histidine operon leader peptide	7968	16222	23577	29432	24705	Operon *hisLGDCBHAFI*. *hisL* encodes the leader peptide of the histidine biosynthesis operon. Activated by ppGpp.
ilvL	*ilvGEDA* operon leader peptide	8081	21763	31086	41425	35876	Operon *ilvLXGMEDA*. *ilvL* encodes the leader peptide for isoleucine and valine biosynthetic operons. Transcription inhibited by leucine responsive Lrp transcription factor.
pheL	leader peptide of chorismate mutase-P-prephenate dehydratase	1598	3983	6112	10093	9562	Operon *pheLA*. Regulates expression of *pheA*.
serA	D-3-phosphoglycerate dehydrogenase	1517	3887	5760	8355	7531	Encodes an enzyme of the L-serine biosynthesis pathway activated by cAMP-CRP and inhibited by leucine responsive Lrp transcription factor.
yaaH	putative regulator, integral membrane protein	1560	3000	4550	7543	6939	Encodes an acetate/succinate symporter.
rpiA	ribosephosphate isomerase, constitutive	1365	1984	3261	5308	4548	Encodes ribose phosphate isomerase. Constitutive promoter.
dapF	diaminopimelate epimerase	2073	3361	4800	7305	6803	Operon *yifL-dapF-yigA-xerC-yigB*. Encodes an enzyme of the lysine biosyntheis pathway.
secM	secretion monitor that regulates SecA translation	3544	5727	7569	8664	7041	Operon *secMA-mutT*.
aroK	shikimate kinase I	2650	5665	7491	9247	7334	Operon *aroKB-damX-dam-rpe-gph-trpS*. *aroK* expression is constitutive. Paralog AroG is inducible during tryptophan limitation and is repressed by TrpR.
glnL	sensory kinase (phosphatase) in two-component regulatory system with GlnG (nitrogen regulator II, NRII)	1684	3601	5522	8115	7172	Operon *glnALG*. cAMP-CRP activates/represses. Fis activates.
aroL	shikimate kinase II	953	2773	4292	6130	5312	Operon *aroL-yaiA-aroM*. TrpR activated by tryptophan represses transcription.
wrbA	flavodoxin-like protein, trp repressor binding protein	2716	4893	7954	11429	9761	Operon *wrbA-yccJ*. CsgD activates transcription by RpoS. Induced during stationary phase growth. Upon *clpP* deletion expression is increased in exponential and stationary phases.
metJ	transcriptional repressor for methionine biosynthesis (MetJ family)	890	1871	2694	4135	3936	Fe^{2+}-Fur represses transcription.

lpd	dihydrolipoamide dehydrogenase, FAD/NAD(P)-binding ; component of 2-oxodehydrogenase and pyruvate complexes; L protein of glycine cleavage complex second part (2nd module)	2646	4350	5404	6141	5065	Operon *pdhR-aceEF-lpd*.
ygjG	putative acetylornithine aminotransferase, PLP-dependent (2nd module)	515	1651	3306	6062	6154	IhfAB regulates transcription. Fis regulates transcription. NtrC activates transcription.
dapA	dihydrodipicolinate synthase	2604	4043	5785	7422	6487	Operon *dapA-bamC*.
icd	isocitrate dehydrogenase in e14 prophage, specific for NADP+ (2nd module)	1149	3644	5739	8515	7940	Facilitates the switch between TCA and glyoxlate pathways. Regulated by phosphorylation. Deletion mutants are sensitive to hydrogen peroxide stress.
glyA	serine hydroxymethyltransferase (2nd module)	1426	3055	4943	10725	10064	Biosynthesis of 5-mTHF, which is used in the biosynthesis of purines, choline, lipids.
yegT	putative MFS family transport protein (2nd module)	1000	2023	3241	4938	4540	Operon *yegTUV*. Uncharacterized protein
leuS	leucine tRNA synthetase (1st module)	1426	2081	3041	4939	4373	Operon *leuS-lptE-holA-nadD-cobC*. Amino-acyl tRNA synthetase.
talB	transaldolase B (2nd module)	2915	3914	6173	9493	9731	Enzyme in the pentose phosphate pathway that links it to glycolysis.
rluE	putative ribosomal large subunit pseudouridine synthase	1240	3211	5411	10786	10351	Operon *rluE-nudJ*. Catalyses the formation of pseudouridine in 23S rRNA necessary for 23S ribosomal RNA function.
talA	transaldolase A (2nd module)	1497	2692	4312	10320	9215	Operon *talA-tktB*. RpoS-activated paralog of TalB
b0360	IS21 protein 1	1720	2547	4188	7896	7620	Operon *insC-1-insCD-1-insD-1*. Repressor of IS-2.
atpI	membrane-bound ATP synthase subunit, F1-F0-type proton-ATPase	1533	3806	5224	7179	6052	ATP-synthase subunit
crp	transcriptional regulator, catabolite activator protein (CAP), cyclic AMP receptor protein (CAMP-binding family), interacts with RNAP	2579	6463	11407	18164	16561	cAMP-CRP can activate or inhibit. Fis inhibits. Cra activates.
pgi	Phospho glucose isomerase	1689	4913	7939	12391	10810	During glycolysis, converts glucose-6-P to fructose-6-P in a reversible reaction.
yeaT	putative transcriptional	569	1450	2942	5404	4654	Required for growth on D-malate as a sole source of carbon and energy.

	regulator (LysR family) (1st module)						
exuR	transcriptional repressor for carbon degradation (GntR family)	2076	3658	6207	8310	7088	Represses transcription of operons responsible for galacturonate and glucuronate catabolism.
yedW	putative transcriptional regulator (OmpR family)	2102	3780	5931	7567	6175	Operon *yedWV*. Response regulator of the YedW-YedV two-component system. Redundant with CusSR system.
sdhC	succinate dehydrogenase , cytochrome b556	1693	9564	14636	20953	18495	Operon *sdhCDAB-sucABCD*. Activated by cAMP-CRP. Activated by Fe^{2+}-Fur. Repressed by Fnr. Subunit of succinate hydrogenase.
cyoA	cytochrome o ubiquinol oxidase subunit II	2799	7183	10500	15415	13584	Operon *cyoABCDE*. Complex regulation. Plays a role in stress-induced mutagenesis.
tnaC	tryptophanase leader peptide	7222	26168	44216	69386	65481	Operon *tnaCAB*. Phosphorylated Tor activates transcription. cAMP-CRP activates transcription.
ygjH	putative tRNA synthetase	2377	6677	11238	17832	17166	Uncharacterized putative tRNA synthetase.
tktA	transketolase 1 thiamin-binding, isozyme	1600	3556	4944	6736	5952	Major transketolase. Mutant cannot utilize pentose as the sole carbon source. *tktA* mutants have increased persister frequency.
gadW	putative transcriptional regulator (AraC/XylS family) (2nd module)	4148	15900	23685	30167	25715	Operon *gadXW*. Complex regulation. RpoS activated. Negative autoregulation. Controls expression of acid resistance genes (*gadA* and *gadBC* operons).
ykfG	CP4-6 prophage; putative DNA repair protein	956	1668	2546	4570	4410	Operon *yafY-ykfB-ykfF-yafX-ykfG-ykfH-yafW-ykfI*. Uncharacterized.
ybcW	DLP12 prophage	1079	2628	4075	7353	6660	Uncharacterized.
dmsA	anaerobic dimethyl sulfoxide reductase, subunit A (1st module)	4034	7916	11867	16260	15168	Activated by Fnr. Repressed by Fis, IhfAB.
gatY	tagatose 6-phosphate aldolase 2, subunit with GatZ	26618	40028	59527	65724	52697	Operon *gatYZABCD*. Activated by cAMP-CRP. Levels increase in response to low pH or acetate stress. Upregulated upon glucose starvation.
fucR	transcriptional activator for L-fucose utilization (DeoR family) (2nd module)	1298	2610	4153	6374	6351	Operon *fucPIKUR*. cAMP-CRP activates. FucR activates.
ytfL	putative hemolysin-related protein (1st module)	803	1930	2982	4507	3719	Inner membrane protein.
yrfF	conserved hypothetical protein	1043	2176	3415	6242	6463	Membrane protein that inhibits the activation of the *rcs* pathway.
aldA	aldehyde dehydrogenase A, NAD-linked	631	1623	3089	5216	4940	cAMP-CRP activates. Fnr represses. Upregulated during adaptation to glucose starvation.
yrbL	unknown CDS	8525	22240	36265	56141	54497	Pho-P activates. BasR-P inhibits. Activated by *evgSA* two-component system.

yhhQ	putative integral membrane protein (1st module)	4056	6740	8540	10727	9191	Predicted inner membrane protein.
mltD	membrane-bound lytic murein transglycosylase D	3816	10135	15418	21636	19936	Outer membrane protein. Lytic meurin transglycolase that breaks down peptidoglycan in the cell wall.
glcC	transcriptional activator for glycolate utilization (GntR family)	1077	2137	3540	6033	5663	Activated by cAMP-CRP. Negatively regulated by Fis. Downregulates glycolate utilization genes.
fldA	flavodoxin 1	2118	4306	6780	8356	6999	Operon *fldA-uof-fur*.
galE	UDP-galactose 4-epimerase (1st module)	1898	3779	5197	6663	5698	Operon *galETKM*. Complex regulation.
mhpR	transcriptional activator for 3-hydroxyphenylpropionate degradation (IclR family)	1398	2714	4791	7758	7423	Operon *mhpR-lacI*. cAMP-CRP activates.
narZ	nitrate reductase 2, alpha subunit (1st module)	1036	2103	2776	4651	4620	Operon *narZYWV*.
ftsH	ATP-dependent zinc-metallo protease (2nd module)	2899	5045	7249	9328	7939	Operon *rlmE-ftsH*. Membrane embedded protease cleaving abberant proteins.
hslV	peptidase component of the HslUV protease	3175	5937	9175	12713	10776	Operon *hslUV*. Peptidase subunit of HslUV protease. Induced by heat shock sigma factor.
yejG	conserved hypothetical protein	3240	9372	14181	19595	16672	Uncharacterized protein.
adhE	multifunctional multimodular AdhE: acetaldehyde-CoA dehydrogenase (1st module)	4103	5448	8160	12716	11722	Activated by Fis and Fnr. Inhibited by Crp and Lrp. Multifunctional, homopolymeric protein. Expression is 10-fold higher under anaerobic conditions.
xseB	exonuclease VII, small subunit	1674	4105	6090	9008	8425	Operon *xseB-ispA-dxs-yajO*. Small subunit of exonuclease 7.
rstA	response regulator (activator) in two-component regulatory system with RstB (OmpR family)	3022	16802	20818	24094	19596	Operon *rstAB*. PhoP-PhoQ activates transcription. *rstAB* is a two component system itself.
yfbV	conserved hypothetical protein	755	2050	3791	6508	7852	Predicted to have protective functions on the chromosome. Becomes abundant after 24 h of incubation (microarray data)
rssB	response regulator involved in protein turnover, controls stability of RpoS (1st module)	1373	2784	4996	9884	9602	Operon *rssAB*. Targets both RpoS and PAP 1 polymerase for degradation. Stationary phase specific induction.
slyB	putative outer membrane lipoprotein	4303	8191	10604	12387	10425	P-PhoP activates or represses transcription. EvgSA activates.
nudE	conserved protein, MutT-like	1884	3905	6309	9749	9836	Hydrolase.

amyA	cytoplasmic alpha-amylase	1327	2839	4857	8148	7249	Cytoplasmic alpha-amylase.
osmC	resistance protein, osmotically inducible	1895	3916	6688	9128	7926	Peroxidase against organic peroxides. Expression is stationary phase specific. Mutants have reduced viability in long-term stationary phase.
bax	conserved hypothetical protein	5531	16229	26248	39314	37629	Uncharacterized protein.
fdoG	formate dehydrogenase-O, major subunit (1st module)	4141	13250	19244	25945	23316	Operon *fdoGHIE*. Subunit of formate dehydrogenase.
yhiD	putative Mg(2+) transport ATPase	1983	5315	7537	10231	9028	Operon *hdeA-hdeB-yhiD*. Complex regulation.
fadB	multifunctional multimodular FadB	366	1597	2830	7433	6790	Operon. *fadBA*. Activated by Fis. Repressed by P-ArcA and FadR. Subunit of the multienzyme complex.
clpB	ATP-dependent protease, Hsp 100, part of novel multi-chaperone system with DnaK, DnaJ, and GrpE (2nd module)	1831	7129	11393	17038	15034	Multifunctional protein. RpoH controls transcription during exponential phase of growth.
yhiI	putative membrane protein	1648	2892	4205	5532	5293	Sigma-28 dependent promoter.
spr	suppresses thermosensitivity of prc mutants at low osmolality (lipoprotein)	3588	7047	8991	12405	10312	Represses DNA damage response genes and DNA replication inhibitors. Represses itself.
sspA	stringent starvation protein A, regulator of transcription	1855	4003	5824	8200	7413	Operon *sspAB*. Negatively regulates H-NS during stationary phase and confers acid tolerance. Starvation induced and alters proteome during long-term starvation.
aspA	aspartate ammonia-lyase (aspartase)	9999	27894	39328	48874	40677	Operon *aspA-dcuA*. Activated by cAMP-CRP and Fnr. Repressed by P-NarL. Converts L-aspartate to fumarate and ammonia.
cfa	cyclopropane fatty acyl phospholipid synthase (unsaturated-phospholipid methyltransferase) (2nd module)	2214	7742	13392	17372	15898	Changes membrane composition by addition of cyclopropane derivates. Promoter is both RpoS and RpoD dependent.
ydfO	Qin prophage;	2591	4024	5679	7628	6892	Operon. *ynfO-ydfO*.
sieB	Rac prophage; phage superinfection exclusion protein	2008	4861	6790	9502	8953	Prevents phage superinfection.
csiE	stationary phase inducible protein	3499	8558	13921	20547	20547	Activated by cAMP-CRP and RpoS.
rof	Rho-binding antiterminator	3279	7539	12174	18243	17923	Operon *yaeP-rof*. Causes antitermination by inhibiting Rho factor activity. Growth rate regulated expression.
def	peptide deformylase	2736	6415	9869	15102	15044	Operon *def-fmt*. Removes formyl group from peptides.
rhsD	RhsD protein in RhsD element (3rd module)	3330	5508	6726	8099	7252	Operon *rhsD-ybbC-ylbH-ybbD*.

zupT	conserved protein	2032	4503	6562	8729	8357	Metal cation transporter.
serW	serine tRNA 5 (duplicate of serX)	5469	26548	34769	43149	39984	1 of 5 serine tRNAs.
yifE	putative LysR type transcriptional regulator with pssR	5714	12585	16094	19130	16858	Plays a role in stress-induced mutagenesis.
yhaJ	putative transcriptional regulator (LysR family)	1615	3286	4799	6795	6587	Uncharacterized protein.
argW	arginine tRNA 5	4236	9965	13059	17260	15633	1 of 7 arginine tRNAs.
pcnB	poly(A) polymerase I	4629	9467	12061	16460	15850	Operon *pcnB-folK*. Polyadenylates 3' ends of mRNAs for subsequent degradation. Levels increase during slow growth rate.
serU	serine tRNA 2	23017	36990	45161	56661	53344	A serine tRNA.
rhoL	rho operon leader peptide	1667	4300	6232	8439	8000	Leader peptide for the *rho* gene.
lpxP	palmitoleoyl-acyl carrier protein (ACP)-dependent acyltransferase, cold induced gene	1921	4065	6899	10764	10987	Cold shock induced. Adds palmitoleate to lipid A in *E. coli* when grown at lower temperatures.
slyD	FKBP-type peptidyl prolyl cis-trans isomerase (rotamase)	5519	10753	15382	21147	20476	Overexpression is toxic.
lpxC	UDP-3-O-acyl N-acetylglucosamine deacetylase	11288	20428	30254	43393	44648	An enzyme of the lipid A synthesis pathway.
frr	ribosome releasing factor	1434	3289	4674	7171	7011	Release factor responsible for disintegration and recycling of ribosomes post translation.
plsB	glycerolphosphate acyltransferase (2nd module)	2311	5064	6613	9270	8892	Participates in persister cell formation in *E. coli*. Catalyses the first reaction of phospholipid biosynthesis.
lepA	GTP-binding elongation factor (1st module)	2311	4234	5909	8139	7771	Operon *lepAB*. Performs a function in translation.
vacJ	lipoprotein precursor	1679	5154	7428	12403	12672	Membrane lipoprotein.
ytfB	conserved protein	1248	5247	7907	12338	12101	*ytfB* overexpression causes filamentous phenotype.
yhfG	conserved hypothetical protein	844	2242	3571	6989	7481	Operon *yhfG-fic-pabA*.
glgS	glycogen biosynthesis, rpoS dependent	5475	18693	31427	48509	48880	Activated by cAMP-CRP. Negative regulation of flagella/motility and promotes biofilm formation.
ygiU	conserved protein	1064	2902	4294	6505	6081	Toxin MqsR that cleaves mRNAs at the GCU sites. Induced upon biofilm formation. Expressed highly in persister cells. Overexpression induces the expression of CspD.
rpsB	30S ribosomal subunit protein S2	6583	15013	18627	21500	19518	Operon *tff-rpsB-tsf*. S2 protein of the ribosome.
ligA	DNA ligase	2302	5530	7414	10992	10638	One of the proteins playing a role in stress-induced mutagenesis.
lysU	lysine tRNA synthetase,	1487	3951	5377	6698	6485	Repressed by Lrp. Inducible lysyl-tRNA synthetase.

	inducible; heat shock protein						
yejL	conserved hypothetical protein	4442	6598	8599	11646	11892	Operon *yejLM*.
hupB	DNA-binding protein HU-beta, NS1 (HU-1)	4910	14663	17971	21972	20309	Activated by cAMP-CRP. Repressed by Fis.
elaB	unknown CDS	939	3239	4885	14207	15263	Paralog of an inner membrane protein which binds to stationary phase ribosomes.
hda	putative chromosomal replication initiator, DnaA-type	585	3287	4491	6764	7331	DNA replication inhibitor. Membrane protein.
pheM	phenylalanyl-tRNA synthetase (pheST) operon leader peptide	8158	16532	22059	28200	27354	Operon *pheMST*. Inhibited by ppGpp.
ybfF	putative enzyme (1st module)	6115	19183	32386	47980	47696	Enzyme with thioesterase activity.
hupA	DNA-binding protein HU-alpha (HU-2)	1512	2723	4491	8895	9543	Activated by cAMP-CRP and Fis.
iscR	putative protein believed to be involved in assembly of Fe-S clusters, DNA-binding domain	1693	2758	4553	8632	9048	Operon *iscRSUA*. Regulates expression of proteins of the Fe-S cluster assembly.
tig	peptidyl-prolyl cis/trans isomerase, trigger factor; a molecular chaperone involved in cell division	3101	4683	6118	8859	8445	Promotes folding of new peptides along with DnaK and GroEL.
uspB	ethanol tolerance protein	917	3240	5340	8861	9019	Stationary phase inducible upon carbon starvation, ethanol stress.
creB	tolerance to colicin E2	803	2343	3669	6487	6562	Operon *creABCD*.
yjjJ	conserved protein	497	1808	3458	7104	7483	Uncharacterized protein.
ychH	unknown CDS	1956	8054	12740	16382	15186	cAMP-CRP activates transcription. Mutant shows increased biofilm formation. Response to hydrogen peroxide and cadmium stress.
cspA	major cold shock protein 7.4, transcription antiterminator of hns,	7217	11871	15850	19142	17038	Cold shock protein.
ppiD	peptidyl-prolyl cis-trans isomerase, for periplasmic folding of outer membrane proteins (1st module)	2249	4800	6723	9086	7880	P-CpxR activates. Reduced level of outer membrane proteins in the mutant.
yobF	unknown CDS	2499	8864	15061	25181	27564	Operon *yobF-cspC*. Stationary phase specific and mediates response to stress conditions.
cspB	Qin prophage; cold shock	353	1557	3129	6596	7060	Cold-shock protein.

	protein; may regulate transcription						
uxuR	transcriptional repressor for uxu operon	1872	4108	5634	9077	8397	Activated by cAMP-CRP. Represses genes for uptake of sugar acids in the absence of sugar acids.
htpX	heat shock protein, integral membrane protein	2246	7119	10349	17241	18888	Activated by RpoH. Promotes degradation of misfolded proteins. Controlled by CpxRA system.
cytR	transcriptional repressor for nucleoside catabolism and recycling (GalR/LacI family)	4417	9977	12364	16324	15458	Controls the expression of genes for transport of ribonucleosides and deoxyribonucleosides.
ileX	isoleucine tRNA 2	3618	5950	6470	9433	8875	tRNA that recognizes the rare codon AUA. Present at relatively low abundance in the cell.
gadX	putative transcriptional regulator (AraC/XylS family)	13715	30463	47376	64245	60991	Operon *gadAXW*. Complex regulation. Controls expression of the acid resistance system.
yafK	conserved protein	2297	6390	8206	13183	13468	In enterroaggregative *E. coli*, required for biofilm formation.
rdoA	conserved hypothetical protein	511	1710	3529	7483	8074	Operon *rdoA-dsbA*. Kinase that phosphorylates at serine and threonine residues. Activated by the Cpx pathway that senses envelope stress.
yafD	unknown CDS	2101	4096	5423	7584	7167	Operon *yafDE*.
pck	phosphoenolpyruvate carboxykinase	3804	6940	9967	12585	11130	Upregulated upon stationary phase entry and during growth on acetate.
yehS	unknown CDS	2103	4975	6917	9029	8651	Uncharacterized protein.
yihN	putative MFS superfamily transport protein (1st module)	1452	6897	12905	26280	27259	Uncharacterized protein of MFS superfamily.
yafL	putative lipoprotein (2nd module)	4883	9278	14017	20330	21095	Uncharacterized protein.
fur	transcriptional repressor of iron transport (Fur family)	708	2265	3739	6020	6151	Operon *fldA-uof-fur*. Activated by cAMP-CRP, oxyR, and soxS. Repressed by Fe^{2+}-Fur.
astC	succinylornithine transaminase, also has acetylornitine transaminase activity, PLP-dependent	780	1063	2192	8782	9384	Operon *astCABDE*. Activated by arg-ArgR. Enzyme of the arginine catabolic pathway (which produces ammonia as a by-product).
dinJ	damage-inducible protein J	4105	8798	13383	23204	25049	Operon *yafQ-dinJ*. Inhibits the RNase activity of YafQ. DinJ is subject to proteolytic cleavage. DinJ represses *cspE* transcription. In colonies, DinJ is expressed lower in the *rpoS* mutant.
evgA	response regulator (activator) in two-component regulatory system with EvgS, regulates multidrug resistance	4852	11512	13238	16121	14558	Operon *evgAS*. Activated by P-EvgA. Repressed by H-NS.

	(LuxR/UhpA family)						
ylaC	unknown CDS	3448	13291	17102	19948	17617	Predicted inner membrane protein.
minC	cell division inhibitor; activated MinC inhibits FtsZ ring formation	3309	9845	14991	22720	22805	Operon *minCDE*. Coordinates cell division by placement of the septum dividing the mother cell.
ycbK	conserved hypothetical protein	5038	13547	18419	29211	29765	Operon *ycbK-gloC*.
tatE	subunit of TatABCE protein export complex	1003	3159	4065	5443	4902	Subunit of a complex that transports proteins out of the cell.
cbpA	curved DNA-binding protein	2380	3827	5107	6609	6231	Operon *cbpAM*. In dimers, binds to curved DNA. Transcription by RpoS during stationary phase entry. Accumulates during long-term stationary phase.
rob	transcriptional activator for resistance to antibiotics, organic solvents and heavy metals (AraC/XylS family) (right origin binding protein) (1st module)	5802	10393	14662	23301	22256	Repressed by Rob, MarA, and SoxS. Promotes antibiotic resistance. Expressed at high levels and activated during stationary phase by RpoS.
pmrD	polymyxin resistance protein B	1722	4445	5745	7613	6802	Confers polymyxin resistance in *Salmonalla*.
ybhQ	conserved protein	2159	6504	9665	14408	14521	Predicted transmembrane protein.
ycgN	conserved hypothetical protein	1489	4232	6089	9701	10000	Uncharacterized protein.
yjjK	putative ABC superfamily (atp_bind) transport protein (1st module)	972	2871	4361	7692	7866	Propells the ribosome into translation elongation in the presence of ATP.
artP	ABC superfamily (atp&memb) arginine transport protein	2289	8951	15122	24509	26332	Operon *artPIQM*. Repressed by arg-ArtP. Predicted subunit of L-arginine transporter.
mglB	ABC superfamily (peri_bind) galactose transport protein (1st module)	3373	15831	22823	29026	29344	Operon *mglBAC*. Binding component galactose transporter.
ymcC	putative synthetase	5075	13706	17977	25099	24805	Operon *gfcBCD*.
dppA	ABC superfamily (peri_bind) dipeptide transport protein (1st module)	3234	13491	25248	47561	51155	Operon *dppABCDF*. Repressed by Fnr. Periplasmid subunit of a dipeptide transporter.
yceP	unknown CDS	3756	6182	7997	11897	11466	Plays a role in biofilm formation. Transcript is abundant during long-term stationary phase.
oppA	ABC superfamily (peri_bind) , oligopeptide	14575	23178	23178	40820	40407	Operon *oppABCDF*. Promotes survival during heat-shock.

	transport protein with chaperone properties						
ycfS	putative enzyme	642	1854	2835	4996	5993	Activated by P-CpxR. Catalyses the attachment of peptidoglycan to the outer membrane.
ydcS	putative ABC superfamily (peri_bind) transport protein	1020	2734	5035	10393	10979	Operon *ydcSTUV-patD*. Binding component of an ABC transporter. Induced during glucose starvation.
ycfD	putative enzyme	1907	4109	5737	7381	7639	Overexpression inhibits colony formation on LB plates.
sraB	small RNA	9125	14673	17830	21877	20150	Small RNA that is expressed during late-stationary phase.
sstT	YgjU DAACS transporter	2751	4842	7091	10000	9525	Serine-threonine symporter.
ppa	inorganic pyrophosphatase	5372	8703	12167	18460	18002	Phosphatase that cleaves pyrophosphate.
focA	FNT family transport protein (formate channel 1) (2nd module)	2185	6061	10676	15678	16395	Operon *focA-pflB*. Activated by cAMP-CRP, Fnr, P-ArcA. Inhibited by P-NarL. Formate transporter that regulates formate levels within the cell.
uspE	conserved protein with adenine nucleotide-binding domain (1st module)	2552	5261	6640	7996	7178	Stationary phase specific induction. Activated by ppGpp and GadX
tonB	energy transducer; uptake of iron, cyanocobalimin; sensitivity to phages, colicins (1st module)	1887	4121	7119	10751	10303	Transporting iron-siderophores and vitamin B12 across outer membrane.
corA	MIT family, Mg2+/Ni2+/Co2+ transport protein (Mg transport system I)	2061	8506	10614	15310	15659	Mg2+ ion transporter.
ppiA	peptidyl-prolyl cis-trans isomerase A (rotamase A)	529	1465	2894	5975	6685	Activated by P-CpxR. Peptidyl-prolyl cis-trans-isomerase
uspF	conserved hypothetical protein with adenine nucleotide-binding domain	4053	13850	21348	36694	39232	Promotes adhesion and reduces motility.
chaC	cation transport regulator	594	1691	3040	6130	6764	Operon *chaCB*. Mutant has reduced swarming motility.
fadL	transport of long-chain fatty acids; sensitivity to phage T2, porin	906	1895	3430	7239	8318	cAMP-CRP and P-PhoP activate transcription. P-OmpR and P-ArcA repress transcription. Outer membrane protein for transport of long chain fatty acids.
b1403	IS21 protein 2	1972	4776	6738	8378	7994	Operon *insC-2-insCD-2-insD-2*.
cspD	similar to CspA but not cold shock induced, nucleic acid-binding domain	4939	28525	55454	89000	Over	Activated by cAMP-CRP and HNS. Inhibited by MqsA. MqsR induces CspD. RpoS independent but stationary phase-specific expression.
rssA	putative transmembrane protein	1191	3007	4876	8778	9412	Operon *rssAB*. Activated by RpoS.

rbsD	D-ribose high-affinity transport system; membrane-associated protein	2946	15733	19494	22816	22073	Activated by cAMP-CRP and inhibited by RbsR. Operon *rbsDACBKR*.
yfgH	putative outer membrane lipoprotein	1161	4287	6441	7928	7403	Operon *yfgHI*. Maintains outer membrane function.
pitB	PiT family, low-affinity phosphate transporter (1st module)	2553	7793	15703	21173	22119	Low affinity high velocity inorganic phosphate transport system.
ydjF	putative transcriptional regulator (DeoR family) (1st module)	2168	4853	6494	8071	7693	Uncharacterized protein.
yahO	unknown CDS	579	1183	2061	5412	6017	RpoS-dependent. Uncharacterized protein.
yehZ	putative ABC superfamily (peri_bind) transport protein (possibly glycine betaine choline transport for osmoprotection)	555	929	2566	5571	7292	Operon *osmF-yehY-yehX-yehW*. Periplasmic component of glycine transporter.
smpA	small membrane protein A	2693	8199	11693	16308	16257	Inhibited by P-CpxR. Lipoprotein. Deletion causes senstivity to rifampicin.
dcd	2'-deoxycytidine 5'-triphosphate deaminase	1789	5279	7039	8717	8815	Catalyses deamination of dCTP to dUTP.
mtr	HAAAP family, tryptophan-specific transport protein	700	6083	8495	10007	9334	High affinity tryptophan transporter. Repressed by trpR.
yfdG	CPS-53 prophage, putative integral membrane protein	2278	5068	6373	7889	7383	Operon *yfdGHI*.
yecH	unknown CDS	839	2181	3796	6167	6882	Uncharacterized protein.
ychF	putative GTP-binding protein	2529	6871	10702	15928	14525	Operon *pth-ychF*. ATPase activity.
hdeA	conserved protein	955	2423	4795	7528	9409	Operon *hdeAB-yhiD*. Complex regulation.
nudC	conserved hypothetical protein ,MutT-like protein	923	4003	5755	7997	9062	Operon *nudC-hemE-nfi*. Removes 5' caps from the RNA molecules.
rmf	ribosome modulation factor (involved in dimerization of 70S ribosomes)	2609	12200	27282	46148	49571	Activated by cAMP-CRP and ppGpp. Contributes to heterogenous gene expression.
yiaF	conserved protein	3735	7919	11445	14047	13795	Uncharacterized protein with polar localization.
rpsO	30S ribosomal subunit protein S15	3375	9777	14252	21639	23596	Activated by Fis and cAMP-CRP. Operon *metY-rimP-nusA-infB-rfbA-truB-rpsO-pnp*.
ygeF	conserved hypothetical protein	2674	8002	11331	14178	13996	Uncharacterized protein with possible role in pathogenesis.
rpsU	30S ribosomal subunit protein S21	7638	18126	24148	31437	29104	Operon *rpsU-dnaG-rpoD*. Repressed by ppGpp-DksA.

ypeA	putative acyltransferase	2076	5635	9016	12867	12758	Operon *ypeA-yfeZ*. Uncharacterized protein.
rpsM	30S ribosomal subunit protein S13	21616	52908	67266	over	over	Operon *rpsMKDAQ*. Repressed by ppGpp-DksA. S13 protein of the 30S subunit. Rmf binds near the S13 subunit.
yajG	putative lipoprotein	3014	8077	10204	11809	10555	Operon *yajG-ampG*.
rsd	regulator of sigma D, has binding activity to the major sigma subunit of RNAP	13106	55544	74639	over	over	Anti-sigma factor to rsd. Stationary phase specific expression.
rpoE	sigma E (sigma 24) factor of RNA polymerase, response to periplasmic stress (TetR/ArcR family)	2042	8958	12482	19906	20462	Operon *rpoE-rseABC*. Inhibited by P-CpxR. Periplasmic and membrane stress response sigma factor. Induced by increasing abundance of misfolded proteins and induced upon entry into stationary phase.
fliY	cysteine binding periplasmic transport protein, sulfate starvation induced	1035	4518	6455	11170	11070	Operon *fliA-fliZ-tcyJ*. Sulphate starvation induced periplasmic binding protein.
ompC	outer membrane protein 1b (ib;c), porin (1st module)	11199	58538	75230	over	over	Activated by P-CpxR, EnvY, P-OmpR. Inhibited by lrp, yjjK. Outer membrane protein.
ihfB	IHF transcriptional dual regulator	3519	6891	9505	14254	14422	Operon *rpsA-ihfB*. Inhibited by ppGpp-DksA.
araC	transcriptional regulator of arabinose catabolism (AraC/XylS family)(2nd module)	924	4028	6217	9312	9430	Activated by cAMP-CRP. Repressed by AraC. Transcriptional regulator.
galP	MFS family, galactose:proton symporter (1st module)	1438	7391	13738	19994	20802	Activated by cAMP-CRP. Repressed by GalR. Member of the MFS transporters.
gmk	guanylate kinase	2696	6732	8814	11813	11939	Nucleotide monophosphate kinase.
clpP	proteolytic subunit of clpA-clpP ATP-dependent serine protease, heat shock protein F21.5	1652	5176	6805	8325	7655	Operon *clpPX*. Proteolytic subunit of the *clpXP* protease.
rnk	regulator of nucleoside diphosphate kinase	1956	4303	6296	9036	8612	Regulator of *ndk* (nucleotide diphosphate kinase).
yqfA	putative transmembrane protein	786	1938	3195	5321	5687	Uncharacterized protein.
yfgA	conserved protein (2nd module)	2588	5216	7839	12038	11562	Inner membrane protein that interacts with MreB and maintains cell shape.
phoH	PhoB-dependent, ATP-binding pho regulon component (2nd module)	3665	7339	10377	12423	11707	Activated by P-PhoB. Induced by phosphate starvation.
pldB	lysophospholipase L(2)	1997	5324	7807	11559	11005	Operon *pldB-yigL*. Phospholipid degradation.

mscS	putative membrane protein, involved in stability of MscS mechanosensitive channel, (1st module)	1477	4042	6114	8981	8757	Mechanosensitive channel induced by RpoS during entry into stationary phase.
ybiH	putative transcriptional represor (TetR/AcrR family)	3460	7263	10229	15865	15613	Operon *ybiH-ybhGHSFR*. Uncharacterized protein.
intF	CP4-6 prophage; putative phage integrase (2nd module)	1383	2731	4475	7692	7428	Uncharacterized protein.
yccA	putative TEGT family transport protein	1993	4889	7949	11919	12118	Membrane protein that interacts with FtsH. Mutant forms less biofilm.
pspE	phage shock protein	1922	5047	8513	12321	11725	Operon *psp*ABCDE. Functions as a thiosulphate transferase.
ybjN	unknown CDS	1881	4984	7098	10595	10077	Operon *ybjC-nfsA-rimK-ybjN*. Mutant has elevated motility.
yhcB	conserved hypothetical protein	1348	2842	4457	5891	5527	Uncharacterized inner-membrane protein.
menG	putative methyltransferase in menaquinone biosynthesis protein	1543	3913	6668	11323	11050	Operon *menAG*. Inhibits ribonuclease E activity. Stationary phase specific expression.
ycgE	putative transcriptional repressor (MerR family) (1st module)	1706	4557	6441	8702	8449	Transcription factor involved in modulation of genes involved in biofilm formation.
moaA	molybdopterin biosynthesis, protein A (1st module)	2696	5918	9105	13332	12854	Operon *moaABCDE*. Differential codon adaptation.
acpP	acyl carrier protein	7325	12306	18260	27424	27593	Operon *fabDG-acpP-fabF*.
lpp	murein lipoprotein, links outer and inner membranes	2035	4569	6622	9254	9128	An abundant lipoprotein in *E. coli*.
yjbJ	unknown CDS	2115	4155	6812	10895	11582	Repressed by FliZ. Induced by RpoS during stationary phase.
bolA	activator of morphogenic pathway (BolA family), important in general stress response	388	2065	4927	10339	11543	RpoS-regulated cell morphology regulator.
ompX	outer membrane protease, receptor for phage OX2	2738	10332	15382	22008	23522	Small outer membrane protein.
yedP	conserved protein with phophatase-like domain	991	5147	8513	14029	15081	Predicted phosphatase.
rcsF	regulator in colanic acid synthesis; overexpression confers mucoid	784	2083	3092	5177	5668	Operon *rcsF-trmD*. Membrane protein that relays signals to RscC.

	phenotype, increases capsule synthesis						
panD	aspartate 1-decarboxylase	2523	8201	12329	16839	16939	Required for pantothenate synthesis.
fadE	putative medium-/long-chain acyl-CoA dehydrogenase (4th module)	407	1731	3152	6273	6179	Repressed by P-ArcA. Acyl-CoA dehydrogenase.
osmE	transcriptiaonal activator of ntrL gene	757	2372	4226	9982	11588	Repressed by Fis. Osmotically induced protein.
yiaG	putative transcriptional regulator	502	1835	4234	8188	9435	Uncharactized protein.
slp	outer membrane protein, induced after carbon starvation	1403	3759	6715	9046	8655	Operon *slp-dctR*. Activated by GadX and YdeO. Repressed by GadW and H-NS. Induced during acid stress.
ompA	outer membrane protein 3a (II*;G;d) (2nd module)	20803	72729	over	over	over	Upregulated during biofilm formation.
ribB	3,4 dihydroxy-2-butanone-4-phosphate synthase	4285	12362	17524	29140	29123	Operon *sroG-ribB*. An enzyme of the riboflavin synthesis pathway.
cld	regulator of length of O-antigen component of lipopolysaccharide chains	4470	11294	14297	19461	19603	Controls the length of O-antigen.

Appendix 7: Primer sequences used in this study

Table 7: Sequences of primers used in this study.

Primer	**Sequence**	Purpose
*clpP*_DF	5'-CGGTACAGCAGGTTTTTTCAATTTTATCCAGGAGACGGAAGTGTAGGCTGGAGCTGCTTC-3'	Disruption forward
*clpP*_DR	5'-CGCCCTGGATAAGTATAGCGGCACAGTTGCGCCTCTGGCAATGGGAATTAGCCATGGTCC-3'	Disruption reverse
*tnaA*_DF	5'-ATGGAAAACTTTAAACATCTCCCTGAACCGTTCCGCATTCGTGTAGGCTGGAGCTGCTTC-3'	Disruption forward
*tnaA*_DR	5'-TTAAACTTCTTTAAGTTTTGCGGTGAAGTGACGCAATACTATGGGAATTAGCCATGGTCC-3'	Disruption reverse
*tnaA*_IF	5'-TTGGCTCTTTCCTGTTAGGC-3'	Confirmation primer
*tnaA*_IR	5'-GGAATGGTTAAACGCAGCAG-3'	Confirmation primer
*tnaA*_FF	5'-TTTGCCCTTCTGTAGCCATC-3'	Confirmation primer
*tnaA*_FR	5'-TGATCAGTCATGATGCCACC-3'	Confirmation primer
*clpP*_FF	5'-GGCGAAAGCGAAAGTGACTG-3'	Confirmation primer
*clpP*_FR	5'-TGGTTGCGAATTTCATGCGG-3'	Confirmation primer
*yliH*_F	5'-CGTCAGCGAAAGCAATCATC-3'	RT-qPCR
*yliH*_R	5'-AGAGCACTCCACTCTTCCTG-3'	RT-qPCR
*rrsA*_F	5'-AGATGAGAATGTGCCTTCGG-3'	RT-qPCR
*rrsA*_R	5'-CGCTGGCAACAAAGGATAAG-3'	RT-qPCR
*dps*_F	5'-AGTGCGCTTGTTTGGTAATC-3'	RT-qPCR
*dps*_R	5'-TATACCCGCAACGATGTCTC-3'	RT-qPCR
*tnaA*_F	5'-TCTGGCGGTAGGTCTGTATG-3'	RT-qPCR
*tnaA*_R	5'-AGACAACGCCAATCTCTTCC-3'	RT-qPCR
*rpoS*_F	5'-ATCTCTTCCGCACTTGGTTC-3'	RT-qPCR
*rpoS*_R	5'-ACCCGTACTATTCGTTTGCC-3'	RT-qPCR
*osmY*_F	5'-TGGCTGTAATGTTGACCTCTG-3'	RT-qPCR
*osmY*_R	5'-ATCGGTGCTCTTGATGTTGTC-3'	RT-qPCR
*katE*_F	5'-TGACGGTGATGTGAAAGGTC-3'	RT-qPCR
*katE*_R	5'-TTCGGGAGTAGAGCAGTTTG-3'	RT-qPCR

Appendix 8: Strains used in this study

Table 8: Strains used in this study

Strains	**Genotype**	**Source/Reference**
A) Strains		
MG1655	Prototrophic *E. coli* K-12, F^- λ^- *rph*	Laboratory stock
MG1655 *ΔtnaA::cat*	As MG1655 but *ΔtnaA::cat*	This study
MG1655 *ΔclpP::cat*	As MG1655 but *ΔclpP::cat*	This study
HS2210	As MG1655 but *ΔrpoS*	(11)
EDL933	O157:H7 substrain EDL933	(9)
EDL933 *ΔrpoS*	As EDL933 but *ΔrpoS*	(9)
ECOR4	Environmental isolate	
ECOR40	Environmental isolate	
ECOR7	Environmental isolate	
ECA03	Environmental isolate	
ABB02	Environmental isolate	
A) Plasmids		
pKD3	Template plasmid for gene disruption, *cat* is flanked by FRT sites that facilitate removal of the resistance cassette.	
pKD46	λ Red recombinase expression plasmid under control of an araC-ParaB inducible promoter.	
pUA66	Low copy number plasmid used for *gfpmut2* reporter gene fusion.	(124)

Standard Operating Procedures

Bacterial growth

1) Streak strains from the -80 °C glycerol stock cultures without thawing onto LB plates containing appropriate antibiotics to isolate single colonies.
2) Innoculate a single colony into 10 ml of LB in a 50 ml Erlenmeyer flask and incubate at 37 °C aerobically with shaking at 200 RPM (Innova 4000, New Brunswick Scientific). *For Gfp screen, strains were grown on Kan (25 µg/ml) plates using 48-pin metal prong. The metal prong is also used for inoculation.*
3) After overnight growth (typically 12 h) subculture 1:10000 into prewarmed 50-ml LB in 250-ml flasks and monitor OD600 using Multiskan Spectrum (Thermo Labsystems). *For Gfp experiments, strains were grown in 150 µl of LB layered with 100 µl of light mineral oil (SIGMA-ALDRICH MKBS7561V) to prevent evaporation.*
4) Exponential phase is defined as OD600 = 0.3 (typically 4 h post inoculation) and early stationary phase as OD600 = 1.5 (~ 5 h 10 min post inoculation).

Polyacrylamide gel electrophoresis

1) Sample collection: At appropriate time points, measure the OD600 of the culture and centrifuge 1.5 ml at 14000 g for 2 minutes.
2) Remove the supernatant with a pipette and wash the cells two times with 1×PBS.
3) Resuspend the cells in Laemmli Sample Buffer (2×Sample Buffer: 12.5 ml of Tris-Cl pH 6.8, 8.7 ml of glycerol, 2.5 ml of β-mercaptoethanol, 10 ml of 10% SDS, 1 ml of 1% bromophenol blue, 15.3 ml of ddH_2O) to a final OD600 of 15 and heat at 100 °C. The protein sample is frozen at -20 degrees °C for long-term storage.
4) To make two 12% polyacrylamide separating gels combine 4 ml of sterile water, 1.5 ml of Tris-Cl pH 8.8, 100 µl of 10% SDS, 4.4 ml of 30% acrylamide solution, 100 µl of ammonium persulphate, and 10 µl of TEMED in a 50 ml falcon tube.
5) Swirl the mixture prior to pipetting between the glass and the spacer plate of the Biorad equipment (Mini-PROTEAN® Tetra Cell, Catalog Numbers 165-8000 and 165-8001) in a casting stand. Wipe the plates with ethanol prior to use.
6) Top off the gel with sterile water and wait 20 – 30 minutes for polymerization to occur. Remove the water and pipette stacking gel (3.6 of sterile water, 630 µl of Tris-HCl pH 6.8, 50 µl of 10% SDS, 660 µl of 30% acrylamide, 50 µl of ammonium persulphate, and 5 µl of TEMED). Insert the comb immediately and wait 20 – 30 minutes for the stacking gel to solidify.
7) Assemble the gels in the gel box and fill with running buffer between the gels and to the appropriate mark in the gel box (10×Running Buffer: 30 g of tris base, 144 g of glycine, 10 g of SDS to 1 L total volume with ddH_2O).
8) Load 5 – 10 µl of the of the heated sample in the wells and run the gel for 30 min at 50 V and 120 min at 100 V using PowerPack 1000/500 (*BIORAD*).

Coomassie staining

1) Soak the gel in staining solution (0.5 g of Coommassie Blue dye, 200 ml of methanol, 50 ml of acetic acid, and 250 ml of sterile water) with slow shaking at 50 RPM for 1 hr.
2) Destain the gel in destaining solution (250 ml of sterile water, 200 ml of methanol, 50 ml of acetic acid) for 30 min, changing the solution every 10 min.
3) Store the gel in 10% acetic acid (this further removes background and hydrates the gel so that the bands become clearer).

Immunoblot

1) Cut a piece of PVDF membrane 9 cm × 7 cm and soak in methanol (in a clean dish) for 5 minutes. Transfer the membrane to cold (stored at 4 °C) transfer buffer (10×Transfer buffer: 6.05 g of tris base, 28.8 g of glycine, 400 ml of methanol, to 2 L with ddH_2O).
2) Also soak in transfer buffer: the polyacrylamide gel with separated proteins, 6 pieces of blotting paper cut 9 cm × 7 cm.
3) Perform the semi-dry transfer using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (*BIORAD* Cat. No. 170-3940): In the order from bottom to top lay down on the apparatus: three blotting papers, PVDF membrane, polyacrylamide gel with separated proteins, and three blotting papers. Use a roller to remove air bubbles between the membrane and the gel and use a tissue paper to remove residual liquid from the apparatus.
4) Connect the electrodes to the power supply and transfer at 20 V for 30 minutes.
5) Blocking to minimize non-specific anti-body binding: Remove the PVDF membrane from the apparatus (remember to keep the same side of the membrane up at all times) and incubate with slow shaking (50 RPM) in 5% milk in TBST (10×TBS-T: 43.5 g of NaCl, 50 ml of 1 M Tris-Cl, pH 8, 2.5 ml of Tween 20, to 500 ml with ddH_2O) for 1 hr.
6) Incubate in 1:10000 dilution of the primary antibody dissolved in 5% milk in TBS-T with slow shaking overnight (12 – 16 h) in a clean plastic dish such that the solution bathes the top surface of the membrane.
7) Wash the primary antibody: Remove the membrane from the antibody and wash 3 times in 10 min intervals with fast shaking (100 RPM) in TBS-T.
8) Incubate the membrane in secondary antibody dissolved in 5% milk in TBS-T with slow shaking for 1 h at room temperature such that the antibody bathes the top surface of the membrane.
9) Wash the secondary antibody three times with TBS-T in 10 min intervals and transfer the membrane to a clean plastic dish.
10) Soak the membrane in 10 ml of the ECL reagents (Amersham, RPN2106) (5 ml of reagent 1 + 5 ml of reagent 2) for one minute and wrap the membrane in packaging film blotting away the excess solution using a blotting paper.
11) Expose the film (Amersham, 28906838) in the dark room for 5 s to 5 min (depending upon the antibody) prior to developing.

Tryptic digestion and MALDI-TOF-MS sample preparation

1) Stain the gel as described above and de-stain as much as possible (until the band of interest is as light as possible).
2) Excise the band from the gel with a clean scalpel and cut into small pieces (approx. 1 mm × 1 mm). Transfer the pieces into a sterile 1.5 ml microfuge tube and de-stain (with 1:1 solution of 100 mM NH_4CO_3 and acetonitrile) further until the pieces become transparent.
3) Add 500 µl of acetonitrile to the pieces for 10 minutes (the pieces will shrink and become opaque). Remove the acetonitrile using a pipette.
4) Add 50 µl of reduction solution (10 mM DTT in 100 mM NH_4CO_3). The gel pieces will absorb the solution and swell. Incubate for 30 min at 56 °C.
5) Remove the solution and repeat step 3.
6) Add 50 µl of alkylation solution (55 mM iodoacetamide in 100 mM NH_4CO_3). The gel pieces will absorb the solution and swell. Incubate for 20 minutes in the dark at room temperature.
7) Remove the solution and repeat step 3.
8) Add enough trypsin (~ 13 ng/µl in 10 mM NH_4CO_3 and 10% acetonitrile) to cover the gel pieces. Allow absorption on ice for ~ 2 h. Let digestion continue for about 16 h at 37 °C. Extract the peptides by acidification of the reaction using trifluoroacetic acid or formic acid. If needed the peptides can be concentrated using ZipTip columns.

Total RNA extraction (using Norgen Biotek, Cat No: 37500)

1) Grow the cells to appropriate density/time and pellet 10^9 cells at 14000 g for 2 min.
2) Discard the supernatant with a pipette and re-suspend the pellet in 100 µl of one mg/ml lysozyme in TE buffer. Incubate for five minutes.
3) Add 300 µl of Buffer RL and vortex to mix thoroughly.
4) Add 200 µl of anhydrous ethanol and vortex to mix thoroughly.
5) Transfer the 600 µl of the mixture to the RNA extraction column and centrifuge at 10000 g for one minute.
6) Add 400 µl of wash solution to the column and centrifuge for one minute.
7) Wash the column twice (a total of three times) with 400 µl of wash solution.
8) Centrifuge for two minutes at 14000 g to dry the column.
9) Add 50 µl of the elution solution directly on top of the column and centrifuge at 200 g for two minutes.
10) Centrifuge at 14000 g for one minute to collect the RNA. RNA can be quantified using Invitrogen Qubit reagent for RNA quantification (Q32855) or Nanodrop 2000 (the former is considered a more accurate method of quantification).

In-solution DNase 1 treatment of RNA

1) For every one µg of RNA 1 U of DNase 1 is used for digestion at 37 °C for 30 min. Combine the reagents as shown in the following table. Scale up the reaction if more RNA is to be digested:

Reagent	Volume/Amount
RNA	1 µg
RNase free DNase 1 (EN0521)	1 U (1 µl)
10 X DNase 1 Buffer	1 µl
RNase free H_2O	Up to 10 µl

Repurification of RNA sample following DNase 1 treatment (using RNA Clean and Concentrator – Zymo Research Cat. No. R1015)

1) Add two volumes of the RNA binding buffer to the DNase 1 treatment reaction mixture and mix well.
2) Add an equal volume of anhydrous ethanol to the reaction mixture and mix well.
3) Add the above mixture to the **Zymo Spin** column and centrifuge at 10000 g for 30 s.
4) Add 400 µl of RNA prep buffer to the column and centrifuge at 10000 g for 30 s.
5) Add 700 µl of RNA wash buffer to the column and centrifuge at 10000 g for two minutes to dry the column.
6) Add the desired amount of RNase-free water to the column and centrifuge at 10000 g for 30 s. Measure RNA concentration.

cDNA synthesis

1) Combine the following reagents in a 0.2 ml PCR tube a

Reagent	**Volume/Amount**
RNA	500 ng
5X Iscript cDNA synthesis mix (*BIORAD*, 170-8890)	4 µl
RNase-free water	To 20 µl

2) Incubate the tube in a thermal cycler under the following conditions: 1) 5 min at 25 °C 2) 30 min at 42 °C 3) 5 min at 85 °C 4) hold at 4 °C/store at 4 °C.

Quantitative PCR (qPCR)

The following are the reaction volumes for a 10-µl reaction (to prepare a master mix, multiply each volume/amount in the following table by the number of reactions to be conducted).

Reagent	Volume/Amount
2×Sso-Fast reaction mix (*BIORAD*, 172-5200)	5.0 µl
Forward primer (25 µM)	0.2 µl
Reverse primer (25 µM)	0.2 µl
cDNA template	1.0 µl
ddH_2O (PCR grade)	To 10.0 µl

Thermal cycling protocol for cDNA quantification: 1) 95 °C for two minutes (initial denaturation) 2) 95 °C for 0.05 s 3) annealing and extension at 55 °C for 10 s. Repeat steps 2), 3), and 4) for a total of 40 times. Record fluorescence after each cycle after step 3). Increase the temperature at 0.05 °C intervals from 65 °C to 95 °C recording fluorescence at each interval increase to generate the melt curve.

One-step PCR-mediated gene inactivation

Preparation of electrocompetent cells and electroporation of disruption product:

1) Inoculate a single colony of K12 MG1655 containing plasmid pKD46 (Amp resistance) into 10 ml of LB with 100 µg/ml of Amp and grow at 30 °C overnight with shaking at 200 RPM.
2) Subculture the cells 1:10, 000 into pre-warmed 500 ml of LB in a 2 L flask containing 100 µg/ml of Amp and 10 mM Arabinose and grow to an OD600 of 0.5 at 30 °C with shaking at 200 RPM.
3) Wash the cells with ice cold 10% glycerol three times prior to concentrating them 100-fold in 10% glycerol.
4) The cells can be used immediately to electroporate the gel purified disruption product or can be flash frozen in liquid nitrogen and stored at – 80 °C for future use.
5) Use 50 µl of the cells and approx. 10 ng of the PCR product in a 0.1 cm cuvette (ice-cold) and electroporate using the Biorad Micropulser (165-2100) EC1 program for bacteria (1.8 kV).
6) Immediately add one milliliter of LB to the cuvette. Transfer the cells into a sterile tube for incubation at 37 °C on a rotating shaking for one hour.
7) Centrifuge the cells at 11000 g for one minute and re-suspend in 300 µl of LB and spread 100 µl on three plates containing 25 µg/ml chloramphenicol.
8) Incubate the plates at 37 °C for 24 h and check for the growth of transformants. Restreak the transformants onto new Chl (25 µg/ml) plates for subsequent DNA extraction for disruption confirmation and making -80 °C glycerol stock cultures.

Polymerase chain reaction

1) Conduct PCR reactions in a 25 µl total volume in a 0.2 ml PCR tube. The following are reagents for a single reaction (multiply each reagent by the number of reactions required to make a master mix):

Reagent	Amount/Volume
ddH_2O	18.6 µl
10X Thermopol Buffer	2.5 µl
10 mM dNTPs	0.5 µl
25 µM forward primer	1.0 µl
25 µM reverse primer	1.0 µl
Taq polymerase (5U/µl)	0.4 µl
DNA template	1.0 µl (20 ng)

2) Thermal cycling conditions: 1) 95 °C for five minutes (initial denaturation) 2) 95 °C for 30 s 3) 58 °C for 30 s (primer annealing) 4) 72 °C for 15 s (extension) 5) Repeat steps 2), 3), 4) for a total for 40 times 6) Final extension at 72 °C for five minutes. The PCR product can be stored at 4 °C.

PCR fragment purification (Using SIGMA-ALDRICH GenElute™ GEL Extraction Kit)

1) Excise the DNA band from the agarose gel using a clean scalpel and transfer to a 1.5 ml microfuge tube (cut as close to the band as possible).
2) Weigh the gel on pieces on the scale and add three volumes of gel solubilisation solution to the gel pieces. Incubate the gel pieces at 50 °C for 10 min until the gel pieces dissolve.
3) Add 500 µl of the column preparation solution to the DNA binding column and centrifuge for one minute at 10000 g.
4) Add one gel volume of isopropanol to the mixture and load the mixture onto the column. Centrifuge for one minute at 10000 g.
5) Add 700 µl of the wash solution to the column and centrifuge for one minute at 10000 g.
6) Add 50 µl of the elution solution to the column and incubate at room temperature for one minute and centrifuge for one minute at 10000 g (the elution solution can be heated to 65 °C to improve the yield).

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