

Investigating the dynamics of expression of persister genes in *Escherichia coli* responding to environmental changes

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Abstract:

Antibiotics have been a one of the many wonders of modern medicine, improving life quality and saving lives on daily basis. Their misuse has caused decreased efficacy worldwide, which is becoming a serious public health threat. An important aspect of decreased antimicrobial efficacy is antibiotic tolerance, of which persisters and Viable but not Culturable (VBNC) cells are important examples: they survive the antibiotic treatment and can give rise to a new population. Therefore, their presence in otherwise susceptible cultures has been implied in recurring infections, threatening safety of food and of water supplies. So far, no obvious markers for these cells have been identified. These tolerant cells appear randomly in bacterial populations, but their existence is also a form of adaptation to environmental challenges, like the presence of antimicrobial drugs. Independently from their susceptibility, all bacterial cells must constantly fine tune their responses to such challenges, and to interact with their surroundings. Signals from the external environment are transmitted across signalling pathways and influence bacterial gene expression. RNA in its different forms, like mRNA, is the link of this communication between the external world and the cell's DNA. For this project, RNA was extracted during the lag phase of *Escherichia coli* BW25113 (wild type) growth in LB medium. Examples of transcriptomics results presented here show that *E. coli* respond to changes in the culture conditions, like medium temperature and pH. This work also presents a protocol for RNA extractions from *E. coli* persisters and VBNC cells in exponential phase, after 3 hours of challenge with ampicillin. They did not yield consistent results in terms of RNA quantity but could become a steppingstone to further investigation of the tolerant phenotypes in *E. coli*.

Table of contents

Abstract	3
Chapter 1: Introduction	9
1.1 Bacteria adapt to their environment	9
1.2 <i>Escherichia coli</i> as a model organism	10
1.3 Public health implications of antibiotic resistance	18
1.4 Microbial genotypic resistance to antibiotics	21
1.5 Microbial phenotypic tolerance to antibiotics	22
1.5.1. Persisters and VBNCs	25
1.5.2. Role of the environment in persisters and VBNCs formation	28
1.5.3. Phenotypic tolerance can lead to genetic resistance	29
1.6 Public health implications of antibiotic tolerance	30
1.7 RNA involved in <i>E. coli</i> gene expression	31
1.7.1 Regulation of mRNA	34
i. Ribonucleases	34
ii. Small RNAs	36
1.7.2 Ribosomal and transfer RNA in <i>E. coli</i>	37
1.7.3 Stochasticity of gene expression	39
1.7.4 Study of mRNA	39
1.8 Work presented in this dissertation	40
Chapter 2: Methods of RNA extraction and quality control from wild type K-12 <i>Escherichia coli</i> strain BW25113	43
2.1 Media, reagents, and growth conditions	44
i. Planktonic <i>E. coli</i> cultures	44
ii. Determining CFU for untreated and treated samples	46
iii. Ampicillin preparation	47

2.2 RNA extractions from time 0 and 1 hour (Qiagen kits)	48
i. Extractions conditions	52
ii. Extractions protocol	53
2.3 RNA extractions from time 1 hour (TRIzol™ Reagent)	55
i. Samples preparation	55
ii. Extraction protocol	56
2.4 RNA purification	57
2.5 Assessment of RNA quantity and quality	60
2.6 Effect of Agencourt® RNA Clean XP® beads	61
2.7 RNA extractions from persisters and VBNCs (Qiagen kits)	62
2.7.1 Spent LB preparation	65
Chapter 3: Results and Discussion	66
3.1 Approximate numbers of <i>E. coli</i> cells used for the experiments	66
3.2 Results of RNA extractions from untreated <i>E. coli</i> in lag phase	69
3.2.1 RNA quality and quantity for samples in lag phase	69
3.2.2 The overall gene expression pattern at times 0 ad 1 hour of culture growth	76
3.3 The most up-regulated genes	79
3.3.1 Response to cold shock	85
3.3.2 Resistance to mildly acidic conditions	87
3.4 The most down-regulated genes	88
3.4.1 Sulphur metabolism	100
3.4.2 Resistance to strong acidic stress	101
3.4.3 Genes associated with the sigma factor RpoS	102
3.5 RNA extractions from persisters and VBNC cells in exponential phase of culture growth	104
3.5.1 Use of spent LB	105
3.5.2 Use of 25×MIC of ampicillin	105

Chapter 4: Conclusions and future developments	106
4.1 Profiling of gene expression of <i>E. coli</i> in lag phase	107
4.2 Investigating gene expression of persisters and VBNC cells	107
4.2.1 Counting of persisters and VBNC cells	108
4.2.2 Investigating of genes of interest	110
4.2.3 Using fluorescent biomarkers	110
Supplementary Materials	113
References	119

List of Figures:

Figure 1: *Escherichia coli* structure.

Figure 2A: *E. coli* doubling times in different types of LB medium determined by Elfving *et al.*, (2004).

Figure 2B: Survival of *E. coli* in LB medium supplemented with NaCl; from Conter 2003.

Figure 3: Bacterial growth curve; from Hall *et al.*, (2013).

Figure 4: A microfluidic device with fluorescently stained *E. coli* trapped in the channels; from Bamford *et al.*, (2017).

Figure 5: Components of flow cytometry instruments.

Figure 6: Time course of bacterial sub-populations' death under antibiotic challenge; from Kaldalu *et al.*, (2016).

Figure 7: *E. coli* cells/ml against time, for treated and untreated liquid cultures determined by Keren *et al.*, (2004a).

Figure 8: RNA transcription and translation.

Figure 9: Factors influencing mRNA lifetime.

Figure 10: *E. coli* small RNAs.

Figure 11: Workflow of RNA sequencing.

Figure 12: Overnight cultures of *E. coli*.

Figure 13: Determining cells/ml of bacteria used for RNA extractions.

Figure 14: Determining the Minimum Inhibitory Concentration (MIC).

Figure 15: Workflow of RNA extractions with Qiagen kits.

Figure 16: Workflow of RNA extractions with TRIzol™ Reagent.

Figure 17: Workflow of RNA purification with Agencourt® RNA Clean XP® Beads.

Figure 18 A-B: Average number of *E. coli* cells used for the experiments.

Figure 18 C: CFU/ml vs OD₆₀₀ of *E. coli* cells used for the experiments.

Figure 19 A-C: Results of RNA extractions from untreated *E. coli* cells at time point 0 hours (Qiagen kits).

Figure 20 A-C: Results of RNA extractions from untreated *E. coli* cells at time point 1 hour (Qiagen kits).

Figure 21: Results of RNA extractions from untreated *E. coli* cells at time point 1 hour (TRIzol™ Reagent).

Figure 22: Frequency distribution of Log₂-fold change vs -log₁₀ SEM for time 0 hours.

Figure 23: Frequency distribution of Log₂-fold change vs -log₁₀ SEM for time 1 hour.

Figure 24: *E. coli* expression of genes involved in cold shock response.

Figure 25: *E. coli* expression of *ompF* and of genes involved in lowered pH response.

Figure 26: *E. coli* expression of genes involved in sulphur metabolism.

Figure 27: *E. coli* expression of genes involved in strong acidic stress.

Figure 28: *E. coli* expression of genes involved in regulation of RpoS.

Figure 29: Counting of persisters and VBNCs, using flow cytometry and persisters assays.

Figure 30: Components of the *tnaC* operon in *E. coli*.

Figure 31: Workflow of potential future experiments using *E. coli tnaC* GFP strain.

List of Tables:

Table 1: Minimum Inhibitory Concentration (MIC) for resistant and susceptible bacteria.

Table 2: Contents and mode of action of reagents in Qiagen RNA extractions kits.

Table 3: Contents and mode of action of additional reagents used for RNA extractions.

Table 4: Reagents used for RNA extractions with TRIzol™ Reagent.

Table 5: Examples of Nano Drop values before and after sample purification.

Table 6: RNA extractions conditions described by **Keren et al., (2004b)** and in this dissertation.

Table 7: *E. coli* BW25113, 15 most up-regulated genes for time point 0 hours.

Table 8: *E. coli* BW25113, 15 most up-regulated genes for time point 1 hour.

Table 9: *E. coli* BW25113, 15 most down-regulated genes for time point 0 hours.

Table 10: *E. coli* BW25113, 15 most down-regulated genes for time point 1 hour.

Table 1S: RNA results for untreated *E. coli* samples (time points 0 and 1 hour), Qiagen extractions.

Table 2S: RNA results for untreated *E. coli* samples (time point 1 hour), TRIzol extraction.

Table 3S: RNA results for *E. coli* persisters and VBNCs in exponential phase (time point 1 hour), Qiagen extractions.

Chapter 1: Introduction

1.1 Bacteria adapt to their environment

No living organism, whether simple or complex, exists as an entity isolated from its surroundings (**Lopez-Maury et al., 2008**). Bacteria have to constantly adapt to fluctuating conditions in their environment (**Vital et al., 2015**) in order to maintain homeostasis (**Lopez-Maury et al., 2008**).

In microbiology, the notion of environment includes different variables that present opportunities and challenges for a bacterial culture, or for single cells.

Scientific literature reports many of such variables and bacterial response strategies; examples include:

- type and abundance of nutrients: The Gram negative bacterium *Escherichia coli*, has specialized chemo receptors to sense nutrients present in the growth medium; it can follow the higher gradient of nutrients using its flagella for movement (**Chantranupong et al., 2015**). It can switch to lactose metabolism after glucose has been depleted from the growth medium: the so-called *diauxic shift* (**Chu and Barnes, 2016**). *E. coli* grows exponentially in optimal conditions, but under starvation it can halt its growth in order to survive (**Bergkessel et al., 2016**).
- oxygen availability: **Hayes et al., (2006)** observed that gene expression of *E. coli* under the same pH conditions changed, depending on oxygen availability in the culture.
- interactions with other microorganisms: In natural habitats, bacteria do not always function as single cells, but communicate via quorum sensing which favours formation of organized biofilms (**Li and Tian, 2012**). **Ratzke and Gore (2018)** examined interactions between four different species of soil bacteria and determined that they influence each other's growth by altering the pH of the medium.

- challenges by antimicrobial agents: Resistance to antibiotics of *Pseudomonas aeruginosa* changed, depending on the sequence of drugs used in a controlled evolution experiment (**Yen and Papin, 2017**).
- interactions with host's immune system: *Mycobacterium tuberculosis* infects host's macrophages and uses them to cross epithelial barrier of the lungs (**Cambier et al., 2014**).
- bacteriophages: Bacteria evolved many defence mechanisms against phages, like avoiding virus' attachment (**Labrie et al., 2010**). CRISPR-cas is a bacterial immune system which interferes with foreign viral DNA the bacterium encountered before (**Marraffini and Sontheimer, 2010**)

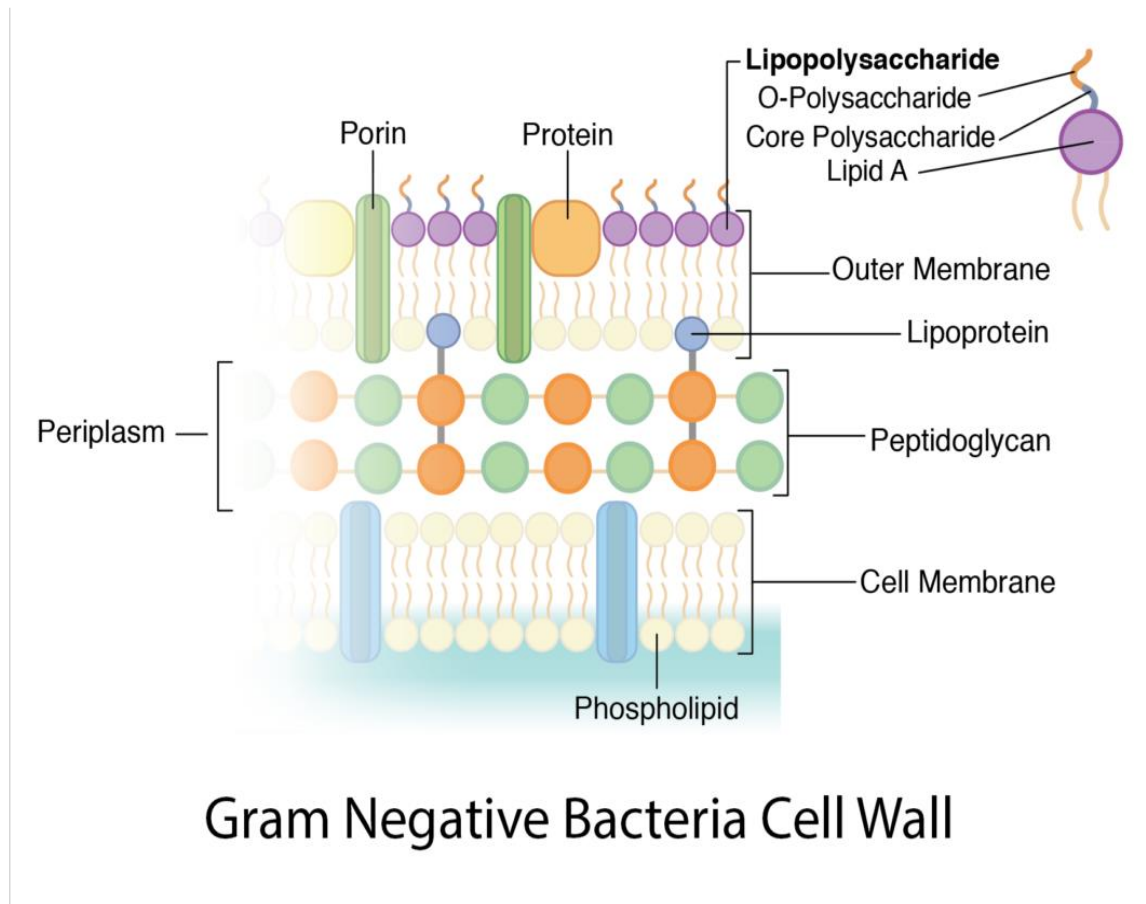
The elements constituting the complex picture of bacterial environment, can be studied in laboratory conditions, where the variables like pH, sugar source and availability, length of exposure to antimicrobial drugs etc. are manipulated by the researcher (**Caglar et al., 2016, Dunne et al., 2017**). This type of studies can be performed using a model organism, like the bacterium *Escherichia coli*, described in the next section.

1.2 *Escherichia coli* as a model organism

Escherichia coli is a collective name for many strains of bacteria, first described in 1885 by Teodor Escherich (**Dunne et al., 2017, Vital et al., 2015**). In the classic Gram staining test performed using crystal violet, they lose the violet colour when treated with alcohol wash, hence their classification as Gram-negative (**Beveridge, 1999**). The reason for the Gram staining results is their layered structure: a thin layer of peptidoglycan between two membranes (inner and outer), divided by periplasmic space (**Solomon et al., 2011**) (Figure 1).

In Gram-positive bacteria, like *Staphylococcus aureus*, the crystal violet stain remains trapped inside the cell by a thick peptidoglycan wall (**Beveridge, 1999**).

Figure 1: Cartoon representation of Gram-negative bacterial envelope structure (From: **Bruslind L. *Microbiology* textbook**; Figure reproduced under Creative Commons Non Commercial-Share Alike 4.0 International License).



E. coli cell shape is usually described as a rod (**Dunne et al., 2017**). However, some environmental conditions can induce changes in cell shape morphology like rounding or elongation (**Bamford et al., 2017**). **Ackermann (2015)** suggested that for rod-shaped microorganisms which actively divide by asymmetrical division, the difference in age of cell components, like the cell wall, contributes to phenotypic diversity in a population.

Commensal non-pathogenic *E. coli* is part of the normal human and animal microbiome (**Dunne et al., 2017**) where it prevents growth of pathogenic

microbes and synthesises vitamins (**The UniProt Consortium, 2018**). Some strains, like Shiga toxin-producing *E. coli* (STEC), are highly pathogenic and cause disease usually spread via contaminated food and water (**CDC, 2019**)

Different *E. coli* strains share many housekeeping genes, but their responses to culture conditions can differ depending on whether they are commensal strains, living in anaerobic conditions of animal intestines, or in an aerobic environment (**Vital et al., 2015**).

E. coli has been studied and used as a model organism for over 100 years (**Dunne et al., 2017**). Today, there are many online tools, which also contain data on *E. coli* strains, available for researchers. Examples include UniProt (**The UniProt Consortium, 2019**), STRING protein database (**Szklarczyk et al., 2019**), or Kyoto Encyclopedia of Genes and Genomes (**KEGG**).

E. coli used in laboratories are often transformed, where plasmids of interest are introduced into bacterial cells, for example expressing Green Fluorescence Protein (GFP) (**Bamford et al., 2017**). Libraries of whole knock-out mutant strains and fluorescent reporter strains can be purchased for research purposes (**Zaslaver et al., 2006**).

A complete sequence of *E. coli* K12 genome was published in 1997 by **Blattner et al.**, while the genome of the BW25113 strain, used in this study, was published by **Grenier et al., in 2014**. Complete genome sequences can be used in transcriptomics studies as reference genomes (**Lowe et al., 2017**).

The non-pathogenic lineages have many advantages as model organisms. They can grow on complex media, like LB (lysogeny broth; sometimes also called Luria Bertani) and LB agar solid medium (**Sezonov et al., 2007**). Lysogeny Broth is composed of:

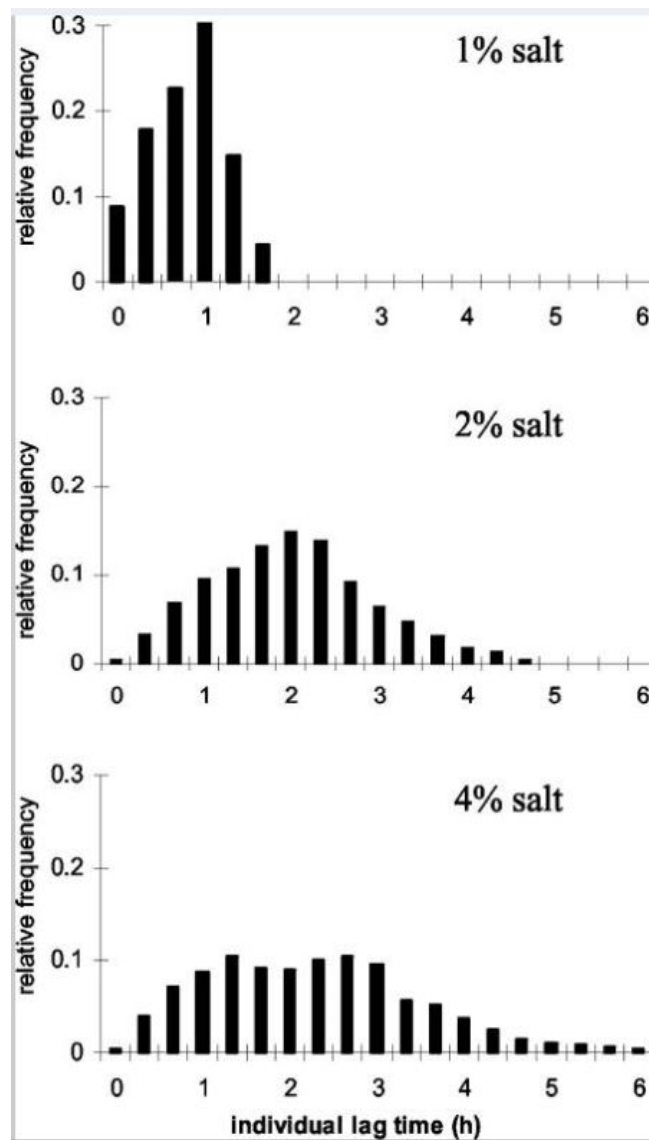
1. tryptone (source of nitrogen and carbon),
2. yeast (provides vitamins and trace elements),
3. sodium chloride (**Merck; Teknova**). Carbon and magnesium have been described as components limiting bacterial growth in this medium (**Sezonov et al., 2007, Christensen et al., 2017**). Since some *E. coli* strains can be sensitive

to high concentrations of sodium chloride (**Merck**), three different modifications of LB are used: Miller (10 g/l of NaCl), Lennox (5 g/l of NaCl), Luria (0.5 g/l of NaCl) (**Teknova**).

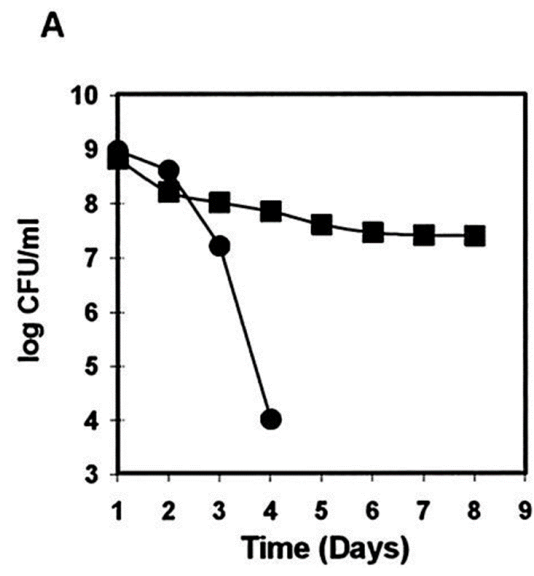
To the best of my knowledge, the literature comparing *E. coli* physiology in different types of LB medium is scarce. **Elfving et al., (2004)** used LB with 1%, 2%, and 4% of NaCl, supplemented with glucose, to grow single *E. coli* cells. They observed that a lower content of salt in the medium was correlated with a shorter lag time before the cells started doubling (Figure 2 A). **Conter (2003)** described that *E. coli* grown in LB medium without any addition of NaCl, lost viability after 4 days, compared to the same strain grown in LB supplemented with 0.4 M of NaCl (23.38 g/L), (Figure 2B). **Baldwin and Kubitschek (1984)** observed the effects of NaCl and sucrose addition on growth and division rates of *E. coli* B/r NC32 grown in liquid cultures at 37°C. They reported an optimal growth at salt concentration of 0.23 M (13.44 g/L). Also, addition of 2% sucrose favoured growth rates, indicating the role of medium osmolarity.

Figure 2: **A)** Frequency of doubling times of *E. coli* MRE C600 grown in a flow chamber, in LB medium (10 g/L of tryptone, 5 g/L of yeast) with different percentages of NaCl per litre and supplemented with glucose (2g/L); (Figure 4 from **Elfving et al., 2004**). **B)** Survival times of *E. coli* MC4100 grown in LB medium without NaCl (circles), and with a supplement of 400 mM NaCl; (Figure 1A from **Conter, 2003**).

A)



B):



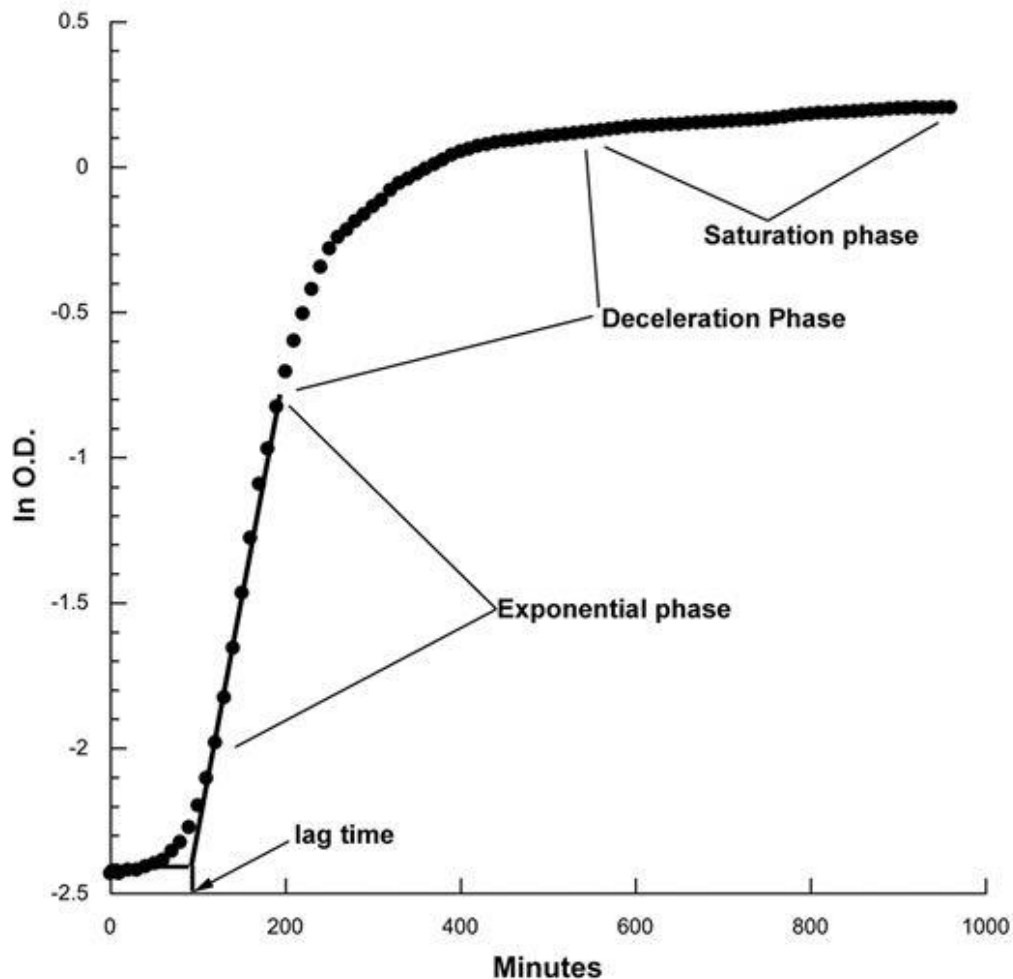
E. coli can also grow on minimal salts media like M9 (**Merck**) which only provide the minimal amounts of nutrients like carbon, nitrogen, amino acids etc. (**Hall et al., 2013**). In favourable conditions, *E. coli* can reproduce by binary fission every 20 minutes, where the population size increases exponentially (**Solomon et al., 2011**).

Its short generation time allows researchers to conduct controlled evolution experiments to better understand how bacteria respond over time to environmental pressures (**Orr, 2005**). For example, **Knöppel et al., (2017)** followed between 500 and 1000 generations of *E. coli* in order to determine how their genome changed over time, while **van Den Bergh et al., (2016)** used controlled evolution techniques to investigate development of drug tolerance in *E. coli*.

When liquid cultures are spotted on agar plates, growing colonies can be assessed and counted without the use of a microscope; glycerol stocks of strains of interest can remain frozen at -80°C prior to the experiments (**AddGene**).

Liquid microbial cultures, for example of *Bacillus cereus*, *Listeria monocytogenes*, or *E. coli*, can be grown at a given temperature, and with an adequate oxygen supply (**Pla et al., 2015**). When optical density values of the culture are plotted against time, we obtain a growth curve which shows distinctive phases (**Hall et al., 2013**); Figure 3.

Figure 3: Schematic representation of phases of bacterial growth. The authors of this figure used the term “deceleration phase” for the gradual transition between the exponential and the stationary phase (here called “saturation phase”). Figure 1 from: **Hall et al., (2013)** “Growth rates made easy”.



1. The lag phase begins when a fresh medium is inoculated with a bacterial colony. Population’s density does not change, but over the course of the phase cells begin to grow (**Shultz and Kishony, 2013**). In absence of environmental pressure like antibiotics, it is a very short phase (**Madar et al., 2013**).

2. It is followed by a stage of exponential growth, where population’s mass doubles at every division cycle (**Navarro Llorens et al., 2010**) and the cell requires an abundant supply of ribosomal RNA (**Bergkessel et al., 2016**). This

stage can only be sustained until the nutrients become scarce, which is what happens in natural environments (**Bergkessel et al., 2016**).

3. During the stationary phase, there is no increase in cells' mass (**Navarro Llorens et al., 2010**), but bacteria remain viable (**Bergkessel et al., 2016**). Aliquots of *E. coli* cells from this stage can be inoculated into fresh medium and revived to enter the lag phase again (**Keren et al., 2004a**). As the stationary phase continues, numbers of viable cells fall, but many can remain alive by using nutrients from dead cells (**Bergkessel et al., 2016**).

4. Over time cells enter a death phase with progressive loss of cells' viability due to accumulation of oxidative damage (**Navarro Llorens et al., 2010**).

Laboratory-adapted *E. coli* strains only represent a small fraction of the bacterial kingdom (**Deutscher, 2015**). However, they have been an important model organism in microbiology for over one hundred years (**Dunne et al., 2017**). For example, they were used to study and produce recombinant proteins like insulin for human use (**Solomon et al., 2009**).

1.3 Public health implications of antibiotic resistance

Antibiotics are compounds which interfere with specific chemical reactions vital for bacterial survival and replication. For example, β -lactams (e.g. ampicillin) target peptidoglycan assembly, chloramphenicol targets bacterial proteins synthesis, quinolones target bacterial DNA replication (**Van Hoek, 2011**), rifampicin targets RNA synthesis (**NCBI, c**). Many antibiotics in use today were originally discovered in nature, like penicillin produced by mould *Penicillium notatum* in 1924 by Alexander Fleming (**van Hoek et al., 2011**). Others, like four generations of quinolones, were synthesized in research laboratories over three decades (**van Hoek et al., 2011**). Bactericidal drugs are lethal for their target cells, e.g. rifampin used in tuberculosis treatment (**NCBI, c**). Bacteriostatic antibiotics, like tetracyclins, limit bacterial growth rate (**van Hoek et al., 2011**).

Modern medicine, as we know it today, has been built on the possibility of preventing and controlling infection. Procedures considered standard nowadays,

like organ transplants or caesarean sections, would become very risky to the patients without antimicrobial drugs. (**Brown and Wright, 2016**).

Antibiotics are also routinely used in chronic diseases like cystic fibrosis (CF), or tuberculosis (TB) which extends patients' lives and improves its quality (**Mulcahy et al., 2010; WHO 2018**).

The importance of antimicrobial drugs can be appreciated by considering high infant mortality rates in poorer countries, like Niger, where many children still today do not survive beyond their 5th birthday (**Maxmen, 2018**).

The World Health Organisation (WHO) recognises public health implications of resistance of different bacteria to particular antibiotics, for example *Pseudomonas aeruginosa* resistant to carbapenems (**Branswell, 2017**), or of multidrug resistant *Mycobacterium tuberculosis* (**WHO, 2018**).

For a susceptible bacterial population to be sterilised, a correct Minimum Inhibitory Concentration (MIC) of a given drug is necessary (**Bamford et al., 2017**), which applies both to *in vitro* cultures as well as to microbial populations in infected tissues (**Cambier et al., 2014**). For resistant strains, MIC values are higher than for the susceptible ones (**Brauner et al., 2016**), Table 1.

Table 1: Examples of MIC values for susceptible and resistant bacterial strains, reported by Centre for Disease Control (CDC) National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) (<https://www.cdc.gov/narms/antibiotics-tested.html> Website accessed 24 August 2019).

Bacterial strains	Antibiotic name	MIC ($\mu\text{g/ml}$) of susceptible strain	MIC ($\mu\text{g/ml}$) of resistant strain
<i>Salmonella</i>			
	Kanamycin	≤ 16	≥ 64
	Ampicillin	≤ 8	≥ 32
<i>E. coli</i>			
	Gentamicin	≤ 4	≥ 16
	Chloramphenicol	≤ 8	≥ 32
<i>Campylobacter</i>			
	Azithromycin	≤ 0.25	≥ 1
	Tetracycline	≤ 1	≥ 4

For antibiotics to be effective, they must reach and act on specific targets within bacterial cells (**Davies and Davies, 2010**). Bacteria defend themselves from the drugs through resistance mechanisms, as described in the following sections.

1.4 Microbial genotypic resistance to antibiotics

Genetic resistance is conferred to bacteria by mutations in their DNA: it is an inheritable property of a microorganism (**Kaldalu et al., 2016**). Horizontal gene transfer allows genes conferring antimicrobial resistance to be exchanged between bacterial strains via plasmids, transposons, or bacteriophages that inject bacterial DNA into new cells they infect (**Soucy et al., 2015**). Resistance genes allow bacterial cells to break down the drugs, to limit their accumulation within cells via efflux pumps, or to use alternative metabolic pathways to those that are targeted by the drugs (**Kester and Fortune, 2014; van Hoek et al., 2011**). Genetic resistance has been recognised only during the past few decades. However, research by **D'Costa et al., (2011)** demonstrated that genes carrying resistance existed in microbial populations thousands of years before Fleming's first discoveries. Research by **Knöppel et al., (2017)**, demonstrated that antibiotic resistance can develop also in the absence of antibiotics in the bacterial environment, as a side effect of adaptation to a particular growth medium (LB, M9 supplemented with glycerol or glucose, Mueller-Hinton broth).

According to **Bengtsson-Palme et al., (2018)** there must be an adaptive advantage for maintaining resistance genes in the genome, in order to balance their fitness cost for the bacterium. One of the factors favouring maintenance of widespread antibiotic resistance in microbial populations is constant environmental pressure, under which the trade-off between fitness cost and resistance, shifts towards the latter.

For the past 70 years, antibiotics have been commonly used on a large scale to prevent and to cure infections both in humans and in farmed animals. They have also been used to enhance cattle growth: practice currently banned in many European countries due to its potential as resistance reservoir (**Levy, 2014**). Antibiotics fed to livestock contaminate the environment through animal urine and manure, which in turn influences microbial communities of soil and plants (**Thanner et al., 2016**). Residues of antibiotics metabolites are also present in foods for human consumption, like milk, meat, or eggs (**Ventola, 2015**). In some parts of the United States, crops are routinely sprayed with antibiotics to prevent

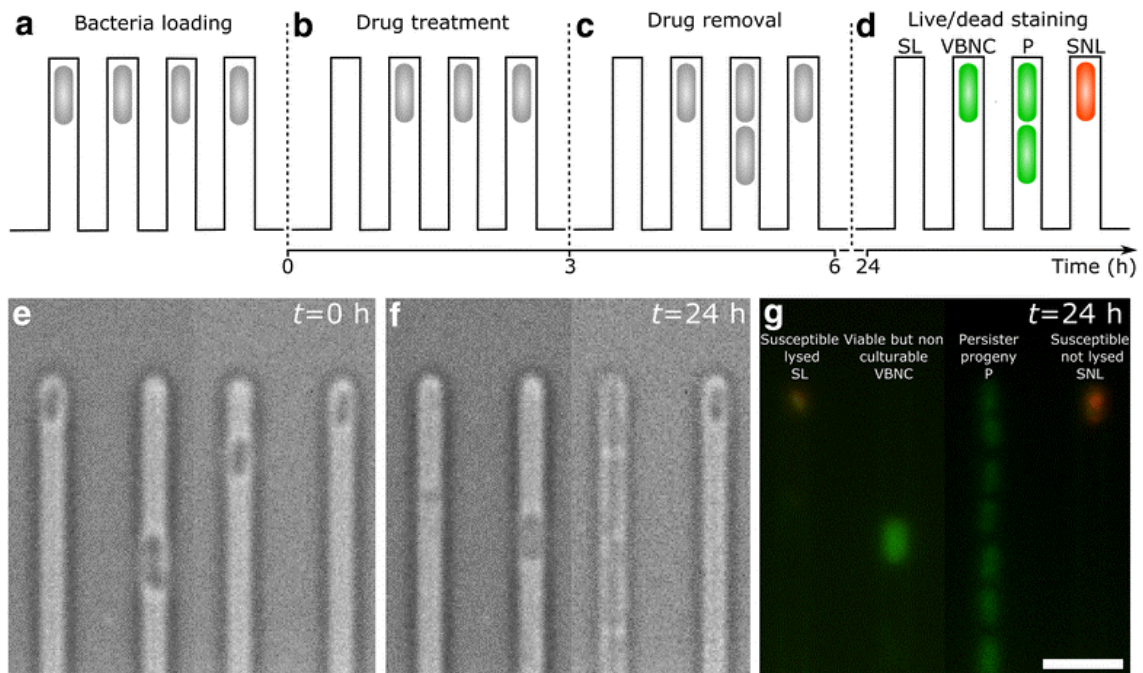
plant infections (**Ventola, 2015**). Drugs are also sprayed on a large scale when diseases emerge, like citrus greening in Florida (US) (**Nature Editorial, 2019**). This drug use and abuse has constantly created an environmental pressure on microbial communities, favouring maintenance of antibiotic resistance in new generations (**Brown and Wright, 2016**) and decreasing its fitness cost for bacteria (**Bengtsson-Palme et al., 2018**).

Evolution of antibiotic resistance is a naturally occurring consequence of environmental pressure on bacteria. Human activities mentioned above, exacerbated the resistance response of microbial populations, leading to serious public health consequences mentioned in section 1.3.

1.5 Microbial phenotypic tolerance to antibiotics

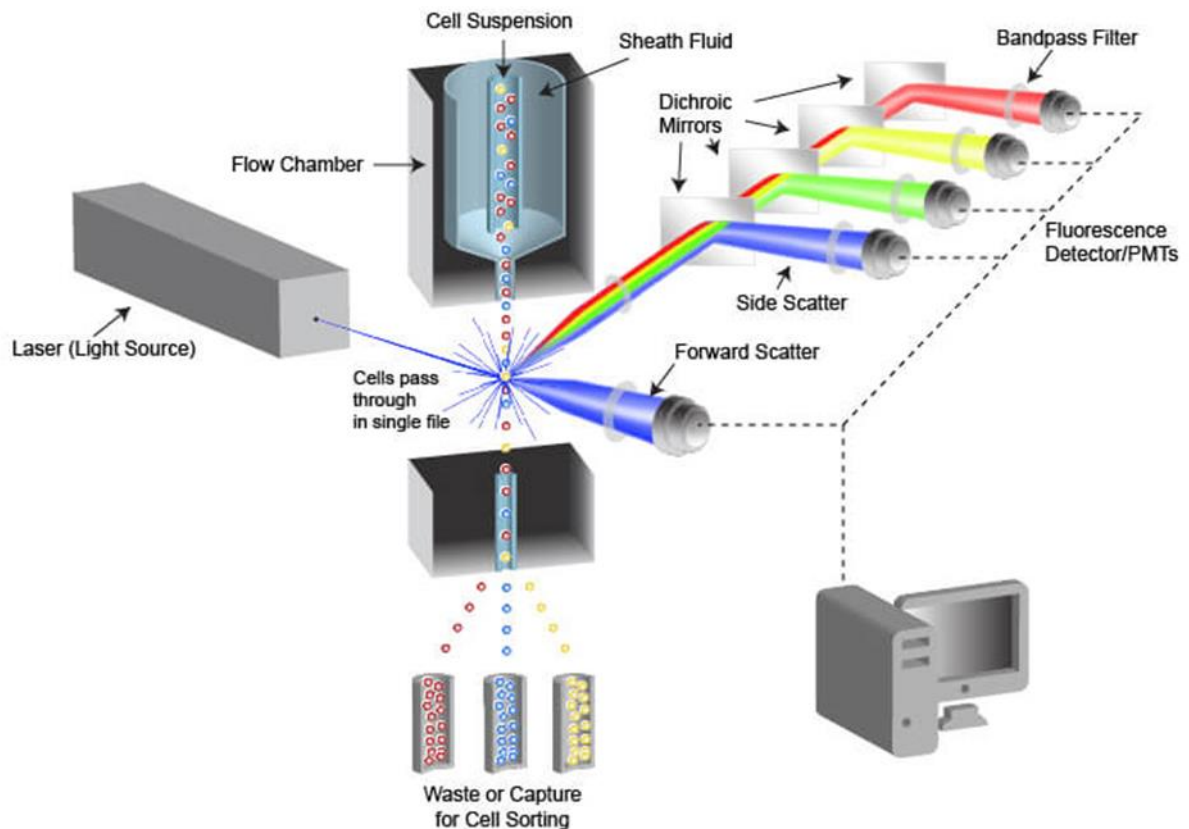
The concept of bacterial phenotypic tolerance to antimicrobial drugs is more subtle than that of genetic resistance. It has been overlooked for a long time, due to the success of new antibiotics development after the Second World War (**Brown and Wright, 2016**), as well as due to insufficient technology available for its study, like microfluidics or flow cytometry (**Hwang et al., 2018**). Microfluidics techniques allow investigation of single microbial cells in small volumes of fluids, which flow within customised microchips (**Elveflow**). The chips are often made of polydimethylsiloxane (PDMS) (**Rusconi et al., 2014**), but can also contain additions like agarose or glass layers to create a desired gradient of a substance, for example of an antibiotic **Li et al., (2014 a)**. The fluids used in the experiments, can either move as constant controlled flow or as droplets (**Elveflow**). Customisation of the chip architecture allows a strict control of the environment of single cells and of small populations of microbes (**Rusconi et al., 2014**). The Mother Machine is an example of a steady-flow microfluidic set up which uses physical confinement and allows to inject and direct single bacteria from the main flow chamber to the thousands of side channels (**Figure 4**). Single cells trapped in the channels can be fluorescently stained and monitored over time under a microscope for responses to external pressures, like presence of antibiotics, or changed nutrients availability in the medium (**Bamford et al., 2017**).

Figure 4: A cartoon and microscopy images of *E. coli* trapped in the channels of a microfluidic device. (Figure 1 from: **Bamford et al., 2017**; reproduced under the Creative Commons Attribution 4.0 International License)



Flow cytometry instruments are composed of a combination of fluidic, optical, and electronic systems, as shown in [Figure 5 \(Cell Signalling Technology\)](#). They use light scattering properties and fluorescence of the analysed sample which is pumped through the instrument in a stream of single cells (**Cell Signalling Technology**). Such techniques allow to quickly count thousands of cells and to assess their viability and morphology (**Thermo Fisher Scientific, a**). Flow Activated Cell Sorting (FACS) instruments also allow to separate sub-populations of cells designated by the researcher by gating and directing the cells into different collection tubes (**Cell Signalling Technology**).

Figure 5: A cartoon schematic representation of the different components of a flow cytometry instrument (Figure from: Overview of Flow Cytometry, Cell Signalling Technology, https://www.cellsignal.co.uk/contents/_/overview-of-flow-cytometry/flow-cytometry-overview).



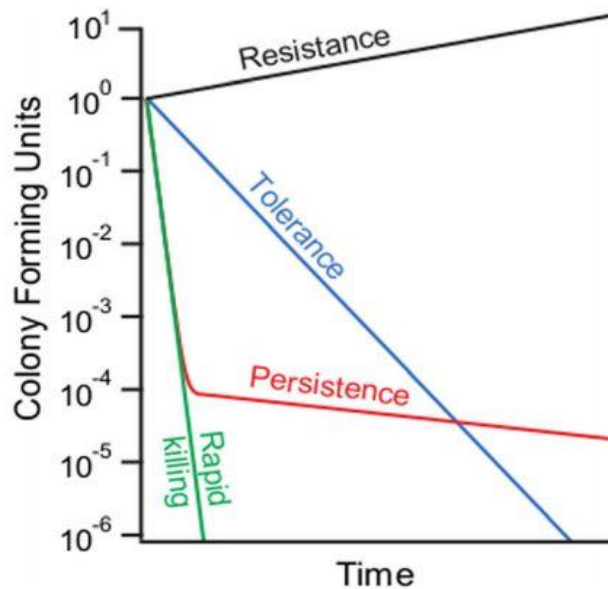
Liquid bacterial cultures originating from an isogenic stock of cells, are not homogeneous (**Kragh et al., 2018**), but are composed of the susceptible and tolerant sub-populations (Section 1.2). **Kragh et al., (2018)** used confocal laser scanning microscopy to investigate formation of cell aggregates conferring increased antibiotic tolerance, of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *E. coli* K12 MG1655 grown in traditional liquid cultures. The authors tested three different inoculation methods of fresh medium for liquid culture: from a frozen stock, from an agar plate, from the overnight culture; the latter is the

method used for this dissertation, section 2.1. Experiments of **Kragh et al., (2018)** demonstrate that bacteria grown in LB medium are not phenotypically homogenous. They also demonstrated that, in case of *E. coli*, inoculation method did not play a role in formation of aggregates. The tolerant sub-populations exist within isogenic populations susceptible to antibiotics, and do not carry the particular genes conferring resistance (**Kaldalu et al., 2016**). MIC of antibiotics required for sterilisation of a culture is the same as for a susceptible strain, but the microbial populations survive for a longer time (**Brauner et al., 2016**). A small fraction of these populations can survive to give rise to new populations at a later time (**Brauner et al., 2016**). According to **Vogwill et al., (2016)**, temporary unfavourable conditions will favour phenotypic tolerance of bacterial populations, as opposed to constant pressures favouring genetic resistance. This consideration is important in clinical context where switching between different drugs regimens is aimed to sterilise infected tissues, at the same time decreasing probability of genetic resistance (**Yen and Papin, 2017**).

1.5.1. Persisters and VBNCs

In 1942, **Hobby et al.** observed that penicillin does not always sterilize completely *Streptococcus* cultures. Under a bactericidal (**Brauner et al., 2016**) antibiotic challenge, most of a susceptible population dies quickly (**Kaldalu et al., 2016**), Figure 6. However, a fraction of up to 1% (in stationary culture), survives and can give rise to another susceptible population, whose small fraction will again survive the antibiotic pressure (**Keren et al., 2004a**). This cycle of death, survival, and re-growth is characteristic of persister cells (**Keren et al., 2004a**).

Figure 6: A graph showing the different subpopulations of bacterial cultures under antibiotic pressure. Persisters show a typical bi-phasic killing pattern: most of the population dies quickly, while for a fraction of the population the killing is very slow. (Figure 1 from **Kaldalu et al., 2016**).



Persisters were first described by **Joseph W. Bigger in the *Lancet* in 1944**. He observed that liquid cultures of *Staphylococcus pyogenes* were not completely sterilized by penicillin, independently on the initial numbers of bacteria. Bigger noticed that the percentage of persisters was always small, and that different culture conditions influenced their final numbers.

Fractions of persisters in bulk populations of *E. coli* can be quantified using microbiology assays, where bacteria are exposed for hours to ampicillin, ofloxacin, or gentamicin, and subsequently re-grown on rich LB agar solid media (**Keren et al., 2004a**). Persister cells are often referred to as dormant cells (**Vega et al., 2012**). However, **Orman and Brynildsen (2013b)** determined that persisters in *E. coli* populations exhibited metabolic activity. Furthermore, their lack of growth before antibiotic challenge, did not determine cells' persistent phenotype.

Persisters are not the only subpopulations present in a culture: in **1982, Xu et al.**, described their innovative methods for analysing of bacterial contamination in

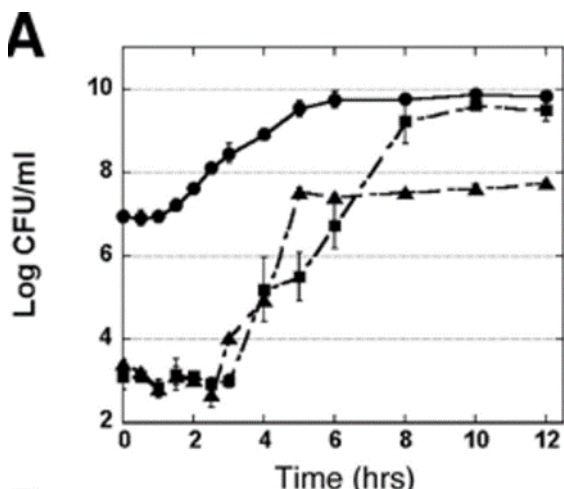
natural water sources. They concluded that traditional plating methods do not take into account for viable cells that do not re-grow on standard media. Today, we refer to them as Viable But Non-Culturable (VBNC) cells (**Orruno et al., 2017**).

Bacteria like *Escherichia coli*, *Mycobacterium tuberculosis*, or *Staphylococcus aureus* can enter both persisters and VBNC states (**Li et al., 2014; Lewis 2007**).

For quantification of VBNC fractions in bulk cultures, flow cytometry and FACS (Section 1.5) can be used in combination with fluorescent Live/Dead staining and with persisters assays (**Orman and Brynildsen, 2013a**).

In genetically identical *E. coli* populations, phenotypically tolerant bacterial cells can be induced through a prolonged challenge with ampicillin (**Keren et al., 2004a**) after which they can be isolated by centrifugation for RNA extraction (**Keren et al., 2004b**). In lack of clear markers of the tolerant phenotypes prior to an environmental challenge, currently the only way of isolating them is a prolonged treatment with bactericidal antibiotics and Colony Forming Units (CFU) counts on agar (**Keren et al., 2004b**). In the experiments of Keren and colleagues (**2004b**), for untreated *E. coli* HM21 (wt), the numbers of cells/ml remained on a steady level (10^7) during the lag phase. They increased at time 4 hours (approaching 10^9), to level off after time 6 hours (approaching values of 10^{10}). In their experiments, numbers of *E. coli* HM21 (wt) treated with ampicillin for 3 hours, decreased by 4 orders of magnitude (Figure 7). As mentioned earlier, this method of estimating bacterial cell numbers does not take in account the presence of VBNCs.

Figure 7: Figure 3A from the paper ‘Persister cells and tolerance to antimicrobials’ by **Keren et al., (2004a)**. Circles are numbers of untreated *E. coli* HM21 (wt), while squares are cell numbers after 3-hour challenge with ampicillin.



1.5.2. Role of the environment in persisters and VBNCs formation

Some environmental stressors have been shown to induce persisters and VBNC cells in growth media. For *Escherichia coli*, exposure to bactericidal antibiotics (e.g. ampicillin) or bacteriostatic antibiotics (e.g. rifampin), can induce persister and VBNC states (**Ayrapetyan et al., 2015; Cui et al., 2018; Orman and Brynildsen 2013b; Kaldalu et al., 2016**). In their investigation of effects of human serum on *Escherichia coli*, **Ayrapetyan et al., (2015)** detected persisters and VBNC cells in the populations after antibiotic challenge. Lack of nutrients, for example in stationary phase of a culture grown in lysogeny broth, is another factor favouring emergence of tolerant phenotypes (**Keren et al., 2004a; Bamford et al., 2017**). **Sezonov et al., (2007)** determined that *E. coli* planktonic cultures growth is limited by depletion of carbon in the medium. In *E. coli*, conditions of scarce nutrients induce a *stringent response* (**Begkessel et al., 2016**). The cell produces an alarmone molecule Guanosine pentaphosphate ((p)ppGpp) (**Bergkessel et al., 2016**), which in turn controls the levels of a sigma factor RpoS (**Spira et al., 2008**), also called σ^{38} or σ^S (**Marschall et al., 1998**). Together, they influence protein synthesis, and have been implied in persisters formation

(**Maissoneuve and Gernes, 2014**) by interfering with replication of DNA and temporary growth arrest (**Wilmaerts et al., 2019**). **Pu and colleagues (2016)** determined that *E. coli* which do not accumulate antibiotics within the cells, are more likely to form persisters. This can happen in cells with either high numbers of the TolC protein, important for efflux systems, or in $\Delta ompF$ and $\Delta ompC$ mutants which have decreased outer membrane permeability.

Mycobacterium tuberculosis and *Pseudomonas aeruginosa* persisters can be induced by exposure of bacteria to limited oxygen supply in their environment (**Bergkessel et al., 2016**), while low temperature contributes to entry into VBNC state in *Vibrio* spp. (**Orruno et al., 2017**).

Bamford et al., (2017) observed that *E. coli* subpopulations of persisters and VBNC co-exist in susceptible isogenic populations, already prior to introduction of environmental pressures. This confirms their status of phenotypically tolerant sub-populations within homogeneous populations.

1.5.3. Phenotypic tolerance can lead to genetic resistance

On a population level, emergence of phenotypically different subpopulations capable of withstanding adverse conditions, is advantageous from an evolutionary point of view (**Vogwill et al., 2016**). Genotypic resistance and phenotypic tolerance could be viewed as separate phenomena with no common points. However, research by **Levin-Reisman et al., (2017)** demonstrated that the cycle of deaths, survival of a fraction of a population, which gives rise to a new population, gives time for genetic resistance to develop.

According to **Vogwill et al., (2016)**, bacteria which survive environmental pressures, respond to them with persistence mechanisms or they develop resistance-encoding genes. This depends on the length of the environmental pressure. Therefore, these two mechanisms promoting survival of a fraction of populations, can be considered as complementary to each other.

1.6 Public health implications of antibiotic tolerance

While genetic resistance is a serious threat, it is important also to consider public health implications of bacterial phenotypically tolerant cells. The ability of persisters to give rise to new populations (**Keren et al., 2004a**) means that a small subset of pathogenic bacteria causing an infection can survive treatment.

Persister cells have been identified in different bacterial strains:

Gram-negative: e.g. *Escherichia coli* causing relapsing Urinary Tract Infections (UTI) (**Lewis, 2007**), *Pseudomonas aeruginosa* causing chronic infections of the airways in Cystic Fibrosis (CF) patients (**Mulcahy et al., 2010**), *Borrelia burgdorferi* causing Lyme disease (**Sharma et al., 2015**).

Gram-positive: e.g. *Staphylococcus aureus* which causes a wide range of infections (**Kim et al., 2018**) and has been investigated by Bigger in the 1940s in the context of persistence (**Bigger, 1944**).

Mycobacteria: e.g. *Mycobacterium tuberculosis* causing its associated disease which is difficult to eradicate (**Keren et al., 2011**).

Biofilms are aggregates of bacterial cells immersed in a protective matrix of proteins (**Ding et al., 2017; Maissoneuve and Gerdes, 2014**), and can coat the surface of medical devices like artificial heart valves. Biofilms have been found to harbour subpopulations of persisters (**Lewis, 2007**) and subpopulations of VBNC (**Ding et al., 2017**).

Similarly to persisters, also VBNCs are not indifferent to human health: in their review, **Li et al., (2014)** mention over 50 pathogenic microorganisms, which can form VBNC cells. Among those, there are many foodborne pathogens. They cause gastrointestinal infections particularly dangerous for children, elderly, or pregnant women; for example, *Campylobacter* spp. (**CDC, 2017**), *Listeria monocytogenes* causing listeriosis (**WHO, 2018**) or *Salmonella* spp. causing salmonellosis (**CDC, 2019**). Their presence in food supply chains and in drinking water is an important public health issue, since VBNC cells can be resuscitated in favourable conditions (**Ding et al., 2017**). Effective assessment of VBNC cells

presence in food and in drinking water need to be in place, as samples with a prevalence of VBNC bacteria could yield false negative results (Li *et al.*, 2014).

1.7 RNA involved in *E. coli* gene expression

How are environmental stimuli, like the previously discussed antimicrobial drugs, linked to gene expression?

For cell's viability and survival, it is imperative to produce enough of given proteins at the right time and rate, which is the final goal of gene expression (Laalami *et al.*, 2013). Signals from the external environment, are perceived by living cells via specialized membrane receptors (Solomon *et al.*, 2011). This in turn activates signalling cascades that feed into up- or down-regulation of certain genes (Vital *et al.*, 2015).

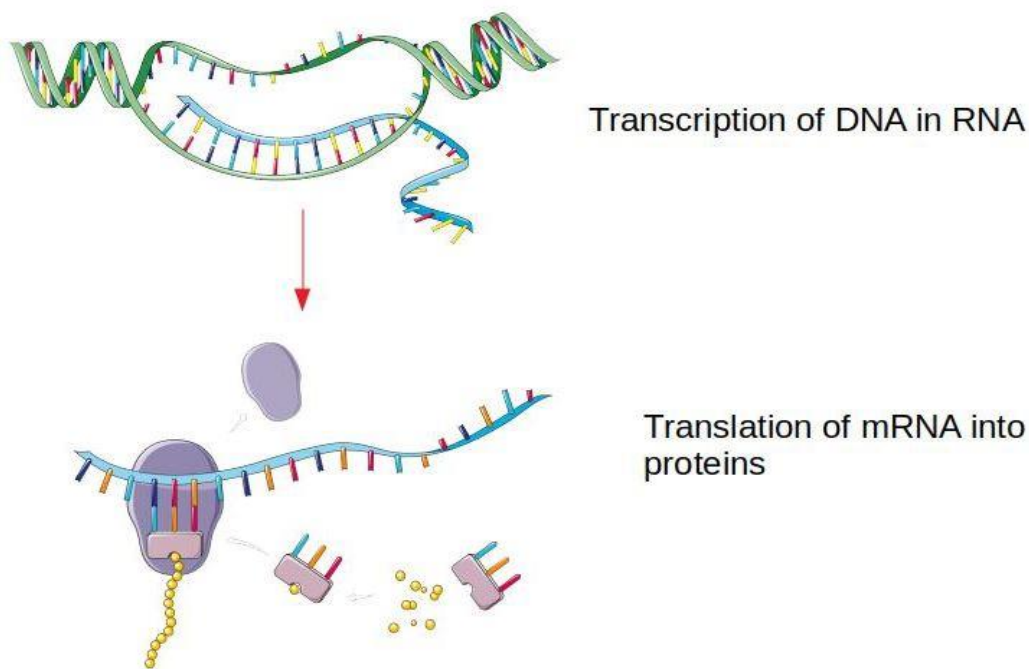
An example of an environmental signalling molecule is indole (Figure 30), a molecule produced from the amino acid tryptophan by the enzyme tryptophanase (Gaimster and Summers, 2015). Research by Vega *et al.*, (2012) demonstrated that the presence of indole in the extracellular space of *E. coli* cells, triggered stress responses pathways and induced formation of persister cells, since pulses of high concentrations of indole inhibit bacterial growth (Gaimster and Summers 2015).

Gene expression, like the one induced by indole, could not take place without RNA: a numerous family of nucleic acids, present in living cells across all kingdoms of life (Solomon *et al.*, 2011). RNA is more ancient than DNA, which is thought to have emerged in the all-RNA world (Buskila *et al.*, 2014). RNAs are usually single-stranded, although their nucleotides can pair to form secondary structures, like hairpin loops, or transfer RNA (tRNA) (Solomon *et al.*, 2011).

The central dogma of biology assumes a flow of genetic information: from DNA to RNA (transcription), then from messenger RNA (mRNA) to protein (translation) (Figure 8). Transfer RNA (tRNA) and ribosomal RNA (rRNA) are subsequently involved in building of the amino acid chain (Buskila *et al.*, 2014). Synthesis of

proteins necessary for maintenance of cell's homeostasis, determine the cell's physiological state (López-Maury *et al.*, 2008).

Figure 8: Schematic representation of transcription and translation. The purple oval shape schematically represents a ribosome moving along mRNA. The square is a schematic representation of tRNA with an associated amino acid. (Cartoons downloaded from *Smart Servier Medical Art* <https://smart.servier.com/>)



The simplified scheme of genetic information flow is true for bacterial cells. However, overall gene expression is regulated by a network of interactions of many different elements involved in transcription, translation, and controlled mRNA degradation (Mackie, 2013).

For bacteria, transcription of DNA and translation of mRNA have traditionally been considered as coupled, meaning that they occur simultaneously (Meyer, 2017). In that case, RNA polymerase (RNAP) moves along a DNA strand, while ribosomes move along the nascent mRNA (Proshkin *et al.*, 2010). An example

of a coupled transcription and translation is the regulation of the tryptophan operon in *E. coli* (**Yanofsky, 1981**)

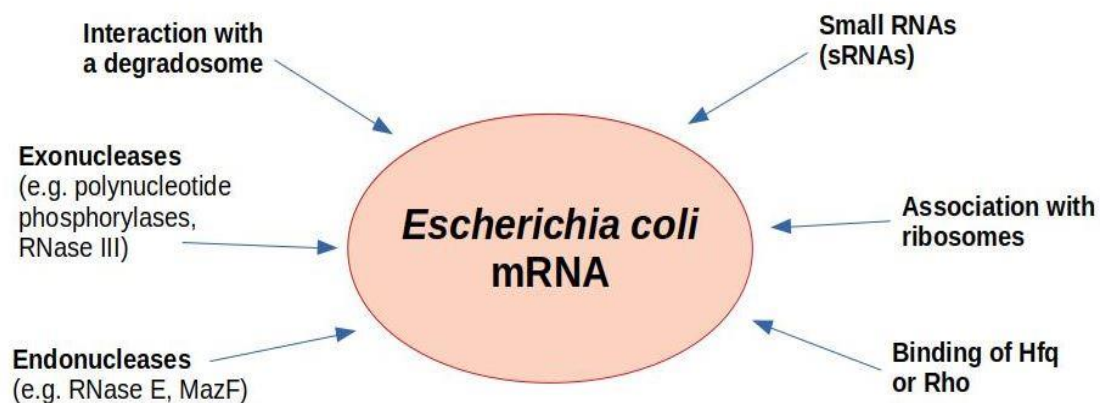
If the new mRNA is not translated, it is targeted by the helicase Rho and by other termination factors (**Leela et al., 2013**) which cause the RNAP to detach from mRNA (**Meyer, 2016**) interrupting transcription. Position of ribosomes and their movement along mRNA or stalling at a given point can protect or expose mRNA to degradation by specialised nucleases (**Meyer, 2016; Hui et al., 2014**). This is an example of a non-nucleolytic mechanism influencing mRNA lifespan (**Laalami et al., 2013**).

mRNA can be prevented from binding to ribosome by interaction with Hfq protein (host factor for phage ϕ replication) and a small RNA (sRNA) (**Vogel and Luisi 2011**).

The literature reports also a different model in which transcription from chromosomal DNA to RNA occurs in the nucleoid (**Mackie, 2013**). After transcription, mRNA moves across cytoplasm by diffusion in order to reach ribosomes (**Mackie, 2013**) mainly located in the cell's periphery (**Laalami et al., 2013**). Most of *E. coli* mRNA undergoes translation after being detached from DNA and RNAP. This view completes the traditional coupling model of transcription and translation in bacteria, which is only true for a small percentage of all transcripts (**Buskila et al., 2014; Laalami et al., 2013**).

1.7.1 Regulation of mRNA

Figure 9: Examples of factors influencing mRNA lifespan in *E. coli*. Interactions of mRNA with ribosomes and proteins Hfq or Rho, have been mentioned in the previous section (1.7). mRNA fate is also influenced by interactions with ribonucleases which, together with other proteins, can form enzymatic complexes like a degradosome. Molecules similar to small RNAs have also been described in *E. coli*, although their role has not been yet established.



i. Ribonucleases

A high mRNA turnover in bacterial cells is one of the most important properties that allows *E. coli* to effectively adjust its levels of gene expression (**Arraino et al., 2010**) and therefore its physiological state (**Vital et al., 2015**). Half-lives of mRNA in *E. coli*, reported in scientific literature, range between 2 and 7 minutes (**Laalami et al., 2014**). This is less than half of the time it takes an *E. coli* cell to duplicate in optimal growth conditions (**Sezonov et al., 2007**).

At the heart of mRNA turnover are endo- and exoribonucleases: enzymes which cleave RNA, often without exhibiting preference for particular nucleotide sequences (**Hui et al., 2014**). They either attack RNA from one end (exonucleases) or from the inside (endonucleases) (**Arraino et al., 2010**). The ability of ribonucleases to break down RNA (nucleolytic RNA inactivation) not only

plays a role in regulation of protein synthesis, but also allows cells to reuse nucleotides (**Laalami et al., 2013**).

RNase E is one of the most important endonucleases in *E. coli* (**Laalami et al., 2013**). It shows a preference for cleaving regions rich in adenine and uracil (**Hui et al., 2014**). mRNA bound to ribosomes is protected from RNase E cleavage, although in some cases mRNA also remains immune to degradation in its free form (**Laalami et al., 2013**).

RNase E is bound to the inner membrane of the bacterial cell (**Mackie, 2013**). Together with enolase, PNPase, and helicase RhlB, it constitutes part of the degradosome (**Hui et al., 2014**). This enzymatic complex is localized at the cell's periphery, where mRNA is cleaved (**Arraino et al., 2010; Mackie, 2013**). RNase E can also enter in interaction with Hfq protein (**Cech et al., 2016**) and a small RNA (sRNA) to form a different type of degradosome (**Arraino et al., 2010**).

Another example of an *E. coli* endoribonuclease is MazF which belongs to one of the ten families of toxin-antitoxin (TA) modules in *E. coli* (**Cho et al., 2017**). In response to environmental stress, like antibiotic exposure, MazF cleaves mRNA as well as the 16 S subunit of rRNA (**Cho et al., 2017, Zorzini 2015**). It interferes with nucleic acids synthesis, which consequently inhibits cell growth and division (**Cho 2017**). The lack of growth can in turn confer a cell the ability of tolerating stress (**Brauner, 2016**)

The toxic effects of MazF are neutralised by a paired antitoxin protein MazE (**Zorzini et al., 2015**). Antitoxins are either proteins or small RNAs, and they remain stable for a shorter time compared to their paired toxins, that are always proteins. (**Unterholzner et al., 2013**).

Cho et al. (2017) observed that *E. coli* cells expressing MazF under exposure to ampicillin, were persisters with an abnormal cell organization. Upon neutralization by MazE, some of the persisters resumed growth, other cells remained in a VBNC state.

After an initial cleavage of mRNA by endoribonucleases, its decay continues by action of exoribonucleases (**Arraino et al., 2010**). They exhibit preference for cleaving RNA from the 3' end or from the 5' end, although the 5' exoribonucleases

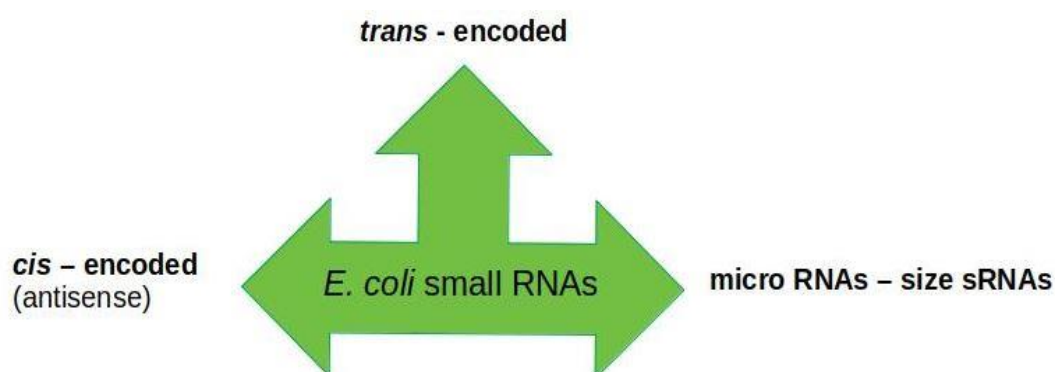
have not been reported in *E. coli* (Hui *et al.*, 2014). An example of exoribonucleases is polynucleotide phosphorylases (PNPs) which only cleave single-stranded RNA, unless RhlB helicase aids in breaking of double-stranded fragments of target RNA (Hui *et al.*, 2014). Transcription of PNPase-encoding gene, is induced in *E. coli* by low environmental temperature (Arraino *et al.*, 2010). RNase III is another example of an exoribonuclease, and it is capable of degrading double stranded RNA (Arraino *et al.*, 2010)

Both RNase E and PNPase autoregulate their own synthesis, by feeding back into the pathway of their translation (Laalami *et al.*, 2013; Meyer, 2017). To maintain cell's viability, nucleolytic activity of RNases has to be controlled (Deutscher, 2015), either by specialized proteins like RraA (regulator of ribonuclease activity A) or RraB (regulator of ribonuclease activity B) that repress RNase E (Laalami *et al.*, 2013), or by other compounds like ATP in case of PNPase (Hui *et al.*, 2014).

Exoribonucleases can also act without the initial endonucleolytic cleavage: they can attack the 3' RNA end which has been polyadenylated by poly(A)polymerase (Arraino *et al.*, 2010).

ii. Small RNAs

Figure 10: Categories of small RNAs described so far in *E. coli* (adapted from: Bloch *et al.*, 2017 and Kang *et al.*, 2013)



Post-transcriptional regulation of gene expression is also performed by small RNAs (sRNAs) which are classified as *trans*- and *cis*- encoded (**Laalami et al., 2013**). Compared to *trans*-encoded sRNAs, *cis*-encoded ones have a better affinity with their target mRNA, as well as a narrower spectrum of targets (**Thomason and Storz, 2010**). *Cis*- encoded RNAs are transcribed from the DNA strand complementary to the one encoding the mRNA target; hence they are also called *antisense* sRNAs (**Thomason and Storz, 2010**). Small RNAs are short sequences of RNA, of up to 250 nucleotides in length; some sRNAs target protein products rather than mRNA (**Michaux et al., 2014**). In *E. coli*, sRNAs can interfere with mRNA binding to the ribosome, and therefore with translation of the message into proteins. Together with Hfq protein, they can also contribute to mRNA cleavage by RNase E (**Hui et al., 2014**). Small RNAs are involved in many processes vital for cell's viability and survival in challenging environments (**Michaux et al., 2014**).

For example, MicF is an antisense small RNA which targets the mRNA encoding OmpF porin, important for osmolarity regulation in *E. coli* cells (**Delihias and Forst, 2001**). Prokaryotic sRNAs can be released from the cells enveloped in Outer Membrane Vesicles (OMV) (**Choi et al., 2017**).

Regulatory small RNAs, about 22 nucleotides long, corresponding to eukaryotic micro RNAs (miRNAs), have been recently identified also in prokaryotes (**Bloch et al., 2017**). In **2013**, **Kang and colleagues** published a study which proves presence of micro RNAs-size sRNAs in *E. coli*. Since eukaryotic miRNAs contribute to gene expression by targeting complementary mRNA (**Choi et al., 2017**), it is possible that this is also the case in bacteria.

1.7.2 Ribosomal and transfer RNA in *E. coli*

Ribosomal RNA (rRNA) and tRNA ([Figure 8](#)), which are the most numerous types of RNAs in growing *E. coli* cells, are examples of untranslated RNAs; they are more stable over time than mRNA (**Li and Deutscher 2008**) and undergo degradation when their quality is compromised (**Deutscher 2006**). rRNA, apart

from its role in translation (Figure 8), can act as a reservoir of nutrients in conditions of starvation (**Li and Deutscher2008**).

Prokaryotic ribosomes, often indicated by its sedimentation coefficient of 70 Svedbergs (S), are constituted of two subunits (**Arraino et al., 2010**):

1. Small subunit 30 S: 16 S rRNA and associated 21 proteins
2. Large subunit 50 S: 23 S rRNA + 5 S rRNA + 33 associated proteins

RNase E together with RNase III and RNase G, cleaves precursors of rRNA into their mature form (**Arraino et al., 2010, Mackie 2013**). Ribosomal RNA constitutes most of cell's RNA (**Lowe et al., 2017**) Endo- and exoribonucleases like RNase E and RNase III also participate in the multi-step maturation of transfer RNA (tRNA) precursors (**Sheperd et al., 2015**). Therefore, ribonucleases exhibit a double function in RNA processing: they contribute to its decay and recycling of nucleotides, as well as to its processing vital for cell's viability.

Since ribonucleases digest RNA and they are present on human skin and in the environment, they are an important factor to consider and to control during RNA extractions in a laboratory to avoid sample degradation (**Ambion**).

Historically, gel electrophoresis with visual assessment of the ratio between bands corresponding to the small and the large ribosomal units, was the method adopted for quantification of RNA quality (**Schroeder, 2006**). A decrease in the ratio between the bands, indicated RNA degradation (**Mueller et al., 2016**). Also today, the ratio between fluorescence values for 23S and 16S ribosomal RNA subunits, is still one of the parameters for determining prokaryotic RNA quality (**Mueller et al., 2016**). However, this ratio is quantified by automated systems like Agilent Bioanalyzer or Agilent Tape Station, and it is expressed as RNA Integrity Number (RIN) (**Schroeder, 2006**).

1.7.3 Stochasticity of gene expression

Within isogenic populations placed in identical environmental conditions, even without any specific stress triggers (**Balaban et al., 2004; Bamford et al., 2017**), the processes of mRNA regulation described above will not yield the same results for all the cells (**Kaern, 2005**). Gene expression is a stochastic process which can be described by mathematical models (**Kaern et al., 2005**): it includes an element of randomness (**Raj and van Oudenaarden, 2008**) in terms of mRNA and protein synthesis (**Ackermann, 2015**). Stress conditions, like lack of nutrients or exposure to antibiotics, increase their numbers (**Balaban, 2011; Brauner et al., 2016**). Research by **Bódi et al. (2017)** on *Saccharomyces cerevisiae* suggests that stochasticity of gene expression influences evolution of populations. For bacteria, as well as for yeast, random fluctuations of protein levels change physiology of the cells, conferring them phenotypes advantageous in a given environment (**Kester and Fortune, 2014**). In the context of persisters, it is often referred to as *bet hedging strategy* (**Maisonneuve and Gernes 2014**). Such a phenotypic plasticity allows subpopulations of cells to survive transient changes in environmental conditions, without the burden of genetic mutations (**Kaern et al., 2005**) or resistance genes gained through horizontal transfer (**Kester and Fortune, 2014**). It is important to remember that a higher fitness in particular stressful conditions is a relative measure: the same sub population may have decreased fitness in an environment without the given stressor (**Bódi et al., 2017**).

1.7.4 Study of mRNA

The total RNA of a cell, including non-coding RNAs (like rRNA or tRNA) and coding mRNA, is called transcriptome (**Manzoni et al., 2018, Croucher and Thomson, 2010**). Transcriptomics is part of the so called -omics tools, that also include genomics, proteomics, epigenomics, and metabolomics (**Manzoni et al., 2018, Hwang et al., 2018**). It is one of the modern approaches to study the connection between the genetic message encoded in DNA and the phenotype of a cell (**Hwang et al., 2018**). RNA sequencing (RNA-seq) is one of the possible

techniques of transcriptome study; the previous technique being microarrays (**Manzoni et al., 2018; Croucher and Thomson, 2010**). RNA-seq is a high-throughput method which can be applied even just to nanograms of RNA (**Lowe et al., 2017**). In eukaryotic cells, it can be used to investigate alternative splicing of mRNA (**Manzoni et al., 2018**) which explains different gene expression in different tissues of the same organism (**Hwang et al., 2018**). It can also be applied in the field of microbiology (**Hör et al., 2018**), for example to study bacterial gene expression in different growth conditions. **Mandlik et al., (2011)** used RNA-seq to investigate gene expression of *Vibrio cholera* in two different animal models. **Wurtzel et al., (2012)** published transcriptomics profile of *Pseudomonas aeruginosa* grown at 28°C and 37°C. RNA-seq can also be applied to both bacterial transcriptome and that of its host cell (dual RNA-seq), which allows a better understanding of their interactions during infection (**Hör et al., 2018**). In the past 10 years it has also been applied to single mammalian cells (scRNA -seq) (**Hwang et al., 2018**). In 2015, **Wang and colleagues** published the single-cell transcriptome of *Synechocystis cyanobacterium*.

Modern laboratory techniques allow researchers to gain valuable insights into the physiology of *E. coli*. Even in such a relatively simple model organism, many elements interact within signalling pathways. Their fine-tuned interplay allows bacteria to interpret signals from their environment and to adjust their physiological state through the appropriate gene expression.

1.8 Work presented in this dissertation

Hypothesis: Bacteria emerging from stationary phase exhibit a significant gene expression reprogramming, which has consequences on bacterial tolerance to antibiotics.

Aim: Determine whether molecular pathways, that are strongly regulated in *E. coli* emerging from stationary phase, play a role in the survival to antibiotics of persister and viable but non culturable cells (VBNC).

Objectives:

1. Investigate gene expression in laboratory-adapted strains of *E. coli* BW2511 (wild type) during lag phase.
2. Investigate gene expression of persisters and VBNC cells in laboratory-adapted strains of *E. coli* BW25113 (wt).

The data obtained for the purpose of this thesis, became part of the manuscript by **Smith et al., (2018)** 'The Culture Environment Influences Both Gene Regulation and Phenotypic Heterogeneity in *Escherichia coli*', published in *Frontiers in Microbiology*. Work presented here mainly concentrates on gene expression of *E. coli* BW25113 (wild type) during the lag phase of growth LB medium containing 5g/L of NaCl. In their natural environment, bacteria usually function in stages of no growth, and rarely are provided the optimal conditions for exponential division (**Shultz and Kishony, 2013**). The lag phase can confer bacteria a transient tolerance to antibiotics (**Brauners et al., 2016**) and is an important factor in determining the shelf life of food (**Shultz and Kishony, 2013**). According to **Rolfe et al., (2011)** the lag phase of bacterial culture growth has been known for years, but did not receive the same attention in the scientific literature as the exponential and the stationary phase, possibly because of the low biomass available when bacteria are in their lag phase of growth.

Specifically, gene expression at different stages of growth in *E. coli* MG1655 has been studied by **Chang et al. (2002)**, but the study focused on the exponential and the stationary phase.

Our work instead sheds new light on the gene expression of a less studied stage of *E. coli* growth. In order to obtain transcriptomic data, RNA was extracted from aliquots of 0.5 ml at times 0 and 1 hour of culture growth, after fresh LB media was inoculated with an aliquot of bacteria from the stationary phase; see Chapter 2 for details. The extractions were performed using Qiagen RNA Protect Reagent, RNeasy micro kit with DNase digest, and clean up with Agencourt® magnetic beads. Only samples having an RNA Integrity Number (RIN) above 8 were taken forward for sequencing. In contrast we report that extractions using TRIzol™ reagent were not successful, with RIN scores below 4.6, which indicates degradation of RNA (**Jahn et al., 2008**). This could be due to insufficient control

of RNases during the process. Exeter University Sequencing Service performed RNA-seq and analysis of the transcriptomic data.

This dissertation also includes a protocol of RNA extractions from persister and VBNC *E. coli* BW25113 (wild type) in early exponential phase of growth (time 4 hours). **Keren et al., (2004a)** used ampicillin and ofloxacin to isolate persisters in bulk liquid *E. coli* cultures and showed that numbers of persisters increase after the first 3 hours of culture growth. Orman and Brynildsen **(2013a)** established that for *E. coli* cultures treated with ampicillin the numbers of VBNC cells were higher than those of persisters. Ampicillin was also the drug of choice for the protocol described here. *E. coli* cells at time 4 hours of culture growth, were incubated for 3 hours in 25 X Minimum Inhibitory Concentration (MIC) of ampicillin. The high concentration of antibiotic combined with a prolonged treatment were applied, in order to ensure sufficient environmental stress together with lysis of as many cells as possible. This was a necessary condition, since antibiotics do not kill bacterial cells instantaneously, even when the microbial population is completely susceptible **(Rossi et al., 2018)**. The attempts of isolating the tolerant cells were performed by centrifugation in order to sediment the unlysed tolerant cells, as described by **Keren et al., (2004b)**. RNA extractions were performed from aliquots of 50 ml of challenged cells, in order to increase the initial volume of starting biological material, as well as from aliquots of 0.5 ml. RNA extractions from samples treated with ampicillin in exponential phase yielded very inconsistent results in terms of the final RNA concentration, and cannot be considered as successful. A possible explanation is contamination of the sample with unlysed cells, with debris of cells burst during the ampicillin treatment.

The protocols described here provide an experimental framework for studying gene expression in wild type *E. coli*. However, it would also be potentially applicable to an *E. coli* strain with fluorescently tagged promoters of the *tnaC* operon **(Zaslaver et al., 2006)** as described in more detail in Chapter 4. Understanding of how the environment shapes *E. coli* gene expression for wild type populations, is the first step towards studying gene expression profiles of the tolerant sub populations isolated from the susceptible bulk of the population.

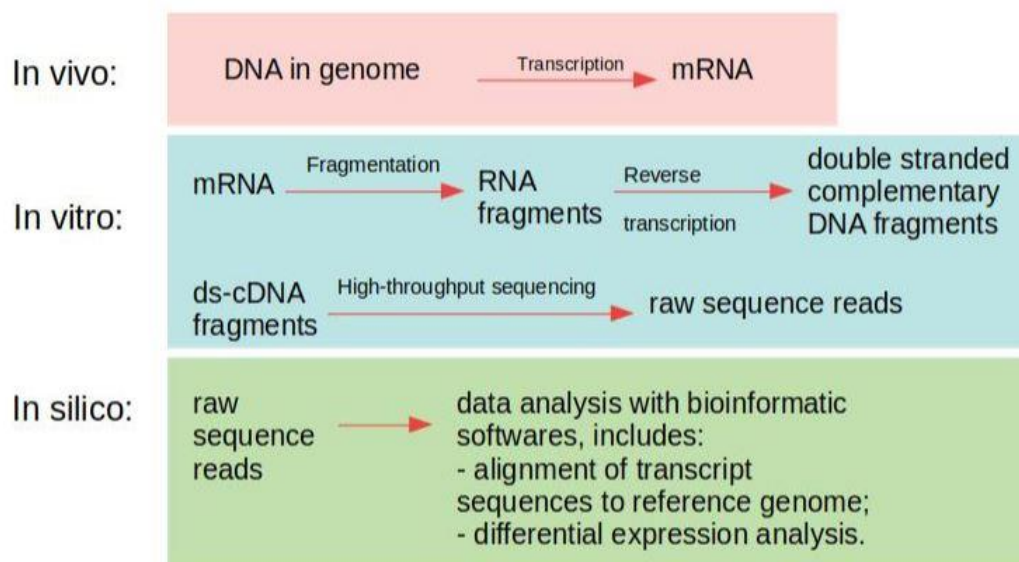
The work presented here is a contribution to a better understanding of how the model organism *E. coli* adapts to its changing environment.

Chapter 2: Methods of RNA extraction and quality control from wild type K-12 *Escherichia coli* strain BW25113

Extraction of good-quality RNA, free of DNA contamination, is the first step of the RNA-seq workflow (Lowe *et al.*, 2017). The next step is fragmentation of the RNA, and reverse transcription of target mRNA into double-stranded complementary DNA (ds-cDNA) (RNA-seqlopedia), [Figure 11](#). A commercial sequencing platform, for example Illumina or PacBio performs the sequencing step (RNA-seqlopedia). The output of a single experiment is a large number of short sequences, therefore data analysis is complex; it involves aligning of the short sequences to a reference genome, as well as subsequent statistical analysis (Lowe *et al.*, 2017).

The results and data for times 0 and 1 hour, obtained with the application of the protocols described here, are presented in Chapter 3.

Figure 11: Workflow for RNA sequencing. Adapted from Figure 4 of **Lowe *et al.*, 2017, *Transcriptomics technologies***. Chapter 2 of this dissertation describes the protocols for mRNA extraction from *E. coli*. Chapter 3 presents some of the transcriptomics results after the data analysis.



2.1 Media, reagents, and growth conditions

Most of the reagents were purchased from Qiagen (Germany), Fisher Scientific (UK), and Sigma-Aldrich (Missouri, US).

i. Planktonic *E. coli* cultures

An effort has been made to ensure consistent growth conditions of the cultures for each experiment. The flasks used to grow the liquid cultures were washed at 70°C, dried at 50°C, and autoclaved prior to use. Bacteria handling was always done by a Bunsen burner and on a bench cleaned with 70% ethanol.

The lysogeny broth (LB) used for liquid culture and agar solid medium was Formedium L-Broth containing 0.5g of NaCl per litre (Formedium, England).

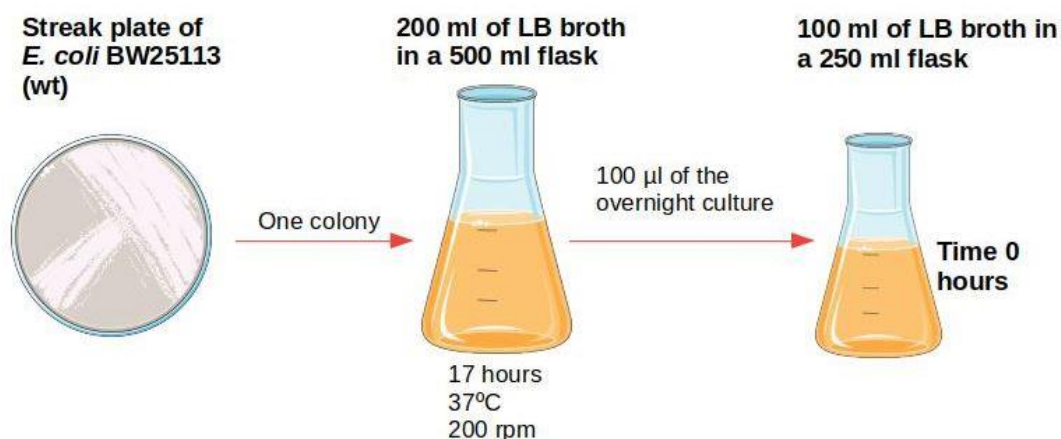
Prior to the experiments, 15.5 g of LB powder were dissolved in 1 L of MilliQ water and autoclaved. Unless a culture was grown at 37°C, all LB media used for the experiments were at room temperature. For agar plates, 6.1 g of the Neogen® Agar No 2 Bacteriological (Neogen, Michigan, USA) were used for every 400 ml

of the same LB that would be used for planktonic growth. The agar plates were poured and dried in a laminar flow hood to ensure their sterility, and stored at 4°C. A fresh streak plate from *E. coli* BW25113 glycerol stock kept at -80°C was made on LB agar at least every 2 weeks and kept at 4°C.

For an overnight culture (Figure 12), one colony was inoculated into 200 ml of LB in a 500 ml conical flask with a foam plug, and grown for 17 hours at 37°C on a shaking platform set to 200 rpm (**Bamford et al., 2017**). After 17 hours, the culture's OD₆₀₀ was assessed. 100 µl of the culture were mixed with by pipetting in a Greiner Bio-One™ semi-micro cuvette containing 900 µl of fresh LB (1:10 dilution), and immediately checked with a Fisherbrand™ Digital Colorimeter 45. To measure OD₆₀₀ for time points 0, 1, and 4 hours, 1 ml of the re-growing culture was pipetted in a Greiner Bio-One™ semi-micro cuvette. The Colorimeter was always first blanked with 1 ml of fresh LB.

To allow *E. coli* to enter a lag phase again, 100 µl of the overnight culture were diluted 1:1000 in 100 ml of fresh LB (at room temperature) in a 250 ml flask, which marked time 0 hours. If further time points of re-growth were required, the flask with a foam plug was placed at 37°C on a shaking platform set to 200 rpm.

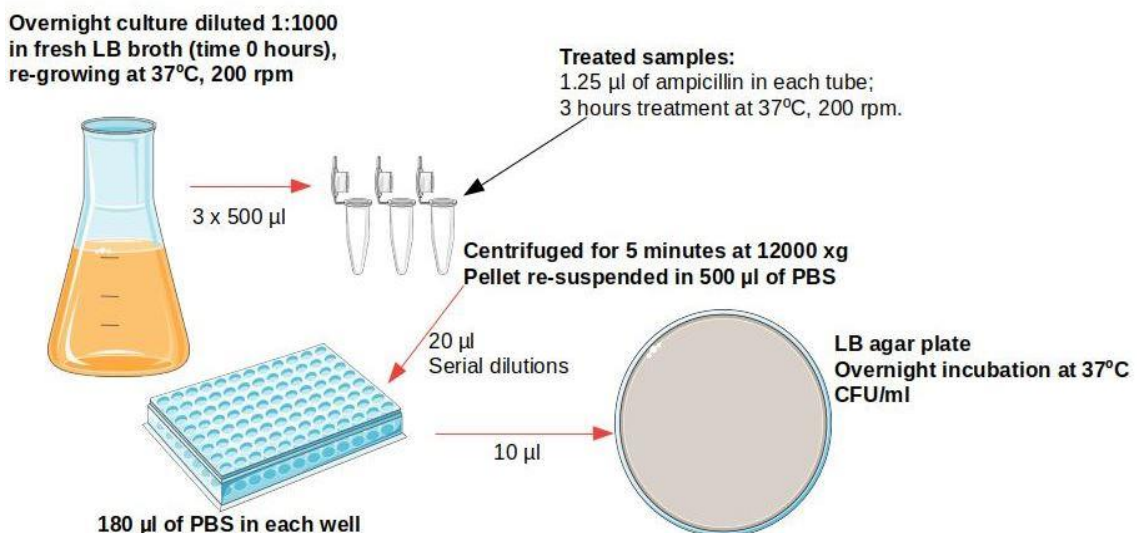
Figure 12: Workflow for preparation of an overnight liquid culture of *E. coli* (cartoons downloaded from *Smart Servier Medical Art* <https://smart.servier.com/>)



ii. Determining CFU for untreated and treated samples

The procedure shown in [Figure 13](#) and described below, was adapted from the protocols for growth stage-dependence of persisters formation of **Keren *et al.*, (2004a)**. For all the procedures, care was taken to maintain the LB agar plates sterile and to dry them in a laminar flow hood cabinet before use.

Figure 13: Workflow for quantifying cells/ml of bacteria used for RNA extractions. Cartoons downloaded from *Smart Servier Medical Art* (<https://smart.servier.com/>)



The CFU were determined both for treated and for untreated samples.

- **Untreated samples:** at given time points, triplicates of 500 µl aliquots of the re-growing diluted overnight culture were spun down with a bench centrifuge at 12000 ×g for 5 minutes. The supernatant was removed with a pipette and the pellet was carefully re-suspended in 500 µl of sterile Phosphate Buffered Saline (PBS).
- **Treated samples:** at time 4 hours, 1.25 µl of fresh ampicillin were added directly to three 500 µl aliquots of bacteria, using the reverse pipetting technique. The tubes were first vortexed vigorously for a few seconds, then

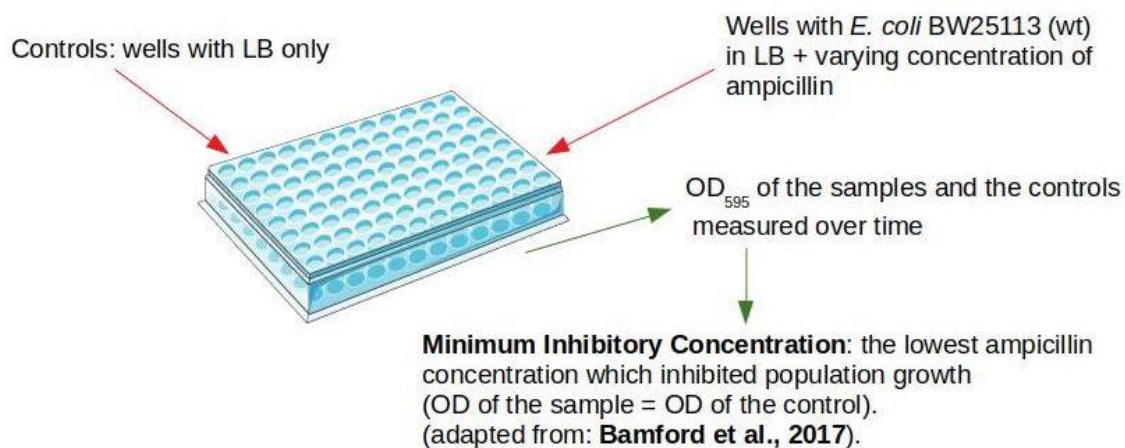
placed on a shaking platform set to 200 rpm at 37°C for a further 3 hours. After that time the centrifugation step was performed (12000 ×g for 5 minutes) and the pellet was re-suspended in 500 µl of sterile Phosphate Buffered Saline (PBS).

180 µl of PBS were aliquoted into 96 wells plate. 20 µl of the re-suspended bacterial pellet were added to the first well: this was the first step of serial 1:10 dilutions. 10 µl of bacteria at dilutions appropriate for a given time point, were spotted on the LB agar. After the plates were dried, they were placed overnight at 37°C. When colonies were visible, they were counted and converted into CFU/ml.

iii. Ampicillin preparation

Ampicillin Sodium Salt powder was dissolved in MiliQ water at a concentration of 50 mg/ml, filtered with a 0.22 µm syringe filter (Minisart™ Sartorius), and frozen at -20°C in order to maintain stock stability as recommended by the manufacturer (Sigma Aldrich). The Minimum Inhibitory Concentration (MIC) for the same stocks of *E. coli* BW25113 (wt) had been determined previously by **Bamford et al., (2017)** and used for the purpose of this thesis. **Bamford et al., (2017)** incubated aliquots of bacteria in a 96-wells plate for 17 hours. They determined the MIC by comparing the Optical density (OD) of the cultures with the OD of the control wells without bacteria, Figure 14.

Figure 14: Determination of Minimum Inhibitory Concentration, as described by **Bamford et al., (2017)** 'Investigating the physiology of viable but non-culturable bacteria by microfluidics and time-lapse microscopy'.



For the experiments described here, a concentration of $25 \times \text{MIC}$ corresponding to $125 \mu\text{g}$ of the stock per 1ml of bacteria was used. Antibiotic aliquots were never older than 7 days in order to avoid the effect of drug degradation, which could potentially lead to lower drug efficacy (**Bamford et al., 2017; Andrews, 2001**). Aliquots of ampicillin were always kept at -20°C and only thawed immediately prior to the experiments. Leftover of the aliquot, or frozen aliquots older than 7 days, were discarded.

2.2 RNA extractions from time 0 and 1 hour (Qiagen kits)

Information provided by Qiagen regarding the composition of the RNeasy Micro Kit buffers is limited, since they are kept confidential by the producer ([Table 2](#)). The spin column used in Qiagen kits selectively binds negatively-charged RNA on the surface of a filter containing silica (**Ali et al., 2017**). This technique of RNA extraction belongs to the so called solid-phase methods; in use from the end of 1970s (**Chacon-Cortes and Griffiths, 2014**).

Table 2. Information provided by Qiagen on reagents and buffers for RNA extractions.

Qiagen reagent or buffer	Composition information disclosed by the producer in the Safety Data Sheets (SDS)	Mode of action of the components disclosed by Qiagen
RNAprotect® Bacteria Reagent	<p>Tetradecyltrimethylammonium bromide (CAS number 1119-97-7); alternative nomenclature: cetrimide, tetradonium bromide; (NCBI, c)</p> <p>Tartaric acid (CAS number 87-69-4)</p> <p>Sodium hydroxide (CAS number 1310-73-2)</p> <p>pH 4.0</p>	<p>Tetradecyltrimethylammonium bromide is a cationic surfactant detergent, frequently used in cosmetics (NCBI, c).</p> <p>Its likely use in RNA extractions is dual:</p> <ol style="list-style-type: none"> 1. Disruption of <i>E. coli</i> cell membranes (Ali et al., 2017). 2. Protection of RNA by binding of the nucleic acid (MacFarlane and Dahle, 1997) and forming a complex of nucleic acid and tetradecyltrimethylammonium salt (Wagner et al., 1969). <p>The low pH of the reagent protects from degradation the phosphodiester bonds of RNA (Bernhardt and Tate, 2012)</p>

Qiagen reagent or buffer	Composition information disclosed by the producer in the Safety Data Sheets (SDS)	Mode of action of the components disclosed by Qiagen
Buffer RLT	<p>Guanidine thiocyanate (CAS number 593-84-0)</p> <p>β-mercaptoethanol (CAS number 60-24-2) added before use. Alternative nomenclature: 2-mercaptoethanol; Thioglycol (NCBI, a)</p>	<p>Guanidine salts are chaotropic agents: they denature the proteins associated with RNA (MacDonald <i>et al.</i>, 1987) by interfering with their hydrogen bonds (Salvi <i>et al.</i>, 2005). They contribute to the cell lysis (Ali <i>et al.</i>, 2017).</p> <p>β-mercaptoethanol increases the effect of guanidine salts (MacDonald <i>et al.</i>, 1987). It protects RNA from nucleases by reducing their disulfide bonds (NCBI, a)</p>
Buffer RW1	<p>Guanidine salt</p> <p>Ethanol.</p>	<p>Guanidine salts: see above.</p> <p>There is no clear indication of ethanol's function in this buffer.</p> <p>RW1 washes off RNA smaller than 200 base pairs (Qiagen, 2014)</p>

Qiagen reagent or buffer	Composition information disclosed by the producer in the Safety Data Sheets (SDS)	Mode of action of the components disclosed by Qiagen
Buffer RDD (part of the DNA digest kit included in the Qiagen RNeasy Micro kit)	No detailed information provided.	Act as a solvent for the DNase I enzyme, supports binding of RNA to the spin column silica membrane (Qiagen, 2014). The Safety Data Sheet does not list any hazardous components.
Buffer RPE	No detailed information provided, ethanol is added before the first use.	This buffer is used in one of the final steps. The ethanol is likely used to precipitate RNA from the solution, before the RNA is dissolved in water (Ali et al., 2017)

Table 3. Additional reagents used in RNA extractions with the Qiagen RNeasy Micro kit.

Reagent	Function in RNA extraction from <i>E. coli</i> cells
Tris EDTA (TE) buffer	EDTA forms bonds with metal ions (is a chelating agent); it affects the integrity of the outer membrane of Gram-negative bacteria (Salazar and Asenjo, 2007)
Lysozyme from chicken egg (c-type) (Ragland and Criss, 2017).	Lysozyme is an enzyme that hydrolyses peptidoglycan polymers of bacterial cell walls, therefore exposing the cells to osmotic stress; the c-type can also form pores in bacterial membranes (Ragland and Criss, 2017).
Proteinase K	Enzyme obtained from fungus <i>Tritirachium album limber</i> ; used to hydrolyse RNases (Sigma Aldrich).

i. Extractions conditions

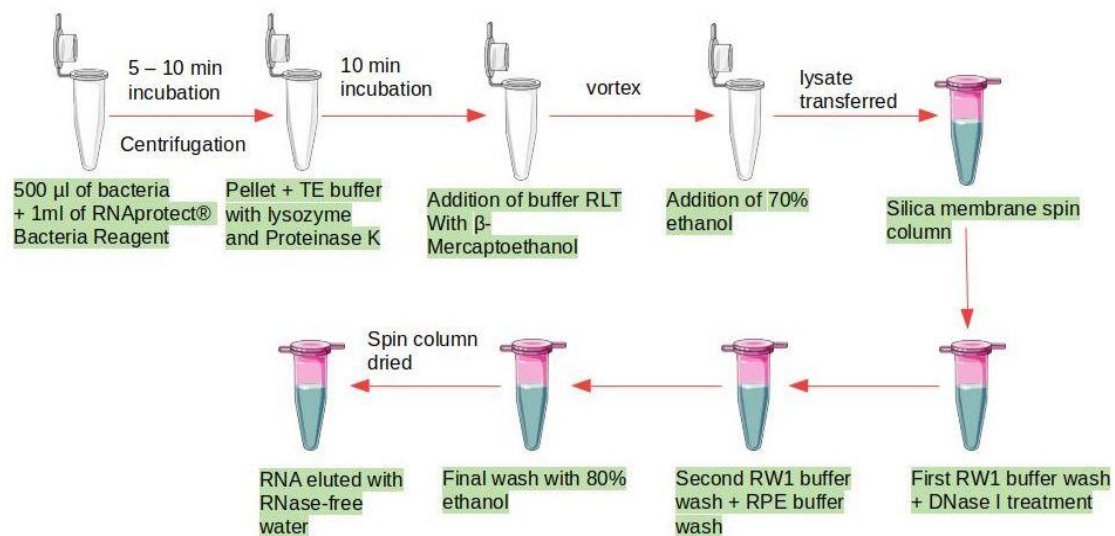
RNA extractions were performed by the Bunsen burner, and only if the OD₆₀₀ of the overnight culture (time point 17 hours) was at least 4.0 ([Figure 18 C](#); Section 3.1) in order to ensure sufficient material. All the centrifugation steps were performed with a bench centrifuge at speeds and with timings suggested in the Qiagen protocols. Care was taken to maintain the bench space as clean as possible (Section 3.2).

RNAprotect® Bacteria Reagent protocol for *E. coli* grown in rich media (**Qiagen, 2015**) was used prior to RNA extraction with RNeasy Micro Kit (**Qiagen, 2014**).

Aliquots of 100 μ l of 1 \times Tris EDTA (TE) buffer with 15 mg/ml of lysozyme from chicken egg were kept at -20°C. 10 μ l of proteinase K was always added to the aliquot just before the experiment. The mixture of buffer RLT containing 10 μ l/ml of β -mercaptoethanol was freshly made at least every 4 weeks.

ii. Extractions protocol

Figure 15: Workflow of RNA extraction from time 0 and 1 hour of *E. coli* culture growth, Qiagen kits (cartoons downloaded from *Smart Servier Medical Art* <https://smart.servier.com/>)



First, RNA was stabilized for 5 to 10 minutes with RNAProtect® Bacteria Reagent: 1ml of the Reagent was pipetted directly to the 0.5 ml aliquot of bacteria.

After centrifugation and discarding of the supernatant, bacterial cells were lysed by addition of 100 μ l of Tris EDTA (TE) buffer with lysozyme and proteinase K. The sample was left to incubate for 10 minutes with 10 seconds of vigorous vortex mixing every 2 minutes. After that, 350 μ l of buffer RLT with β -mercaptoethanol (alternative name: 2-Mercaptoethanol) (**NCBI, a**) were added to the tube and mixed by vortexing.

RNA was purified and eluted following the Qiagen Quick Start protocol of the RNeasy Micro kit. In the steps where the flow-through of the spin column had to be discarded, it was done by pipetting the content out of the collection tube, in order to limit contamination of the silica membrane with waste.

One volume of 70% ethanol was added to the sample and mixed by gently pipetting up and down. An RNeasy micro spin column is made of two parts: the filter with a flip cap (maximum volume is 700 μ l) and the collection tube. Up to 700 μ l of the lysate at a time was transferred from the Eppendorf tube to the spin column and centrifuged, which caused the lysate to be filtered into the collection tube. Then the rest of the lysate was transferred and centrifuged. The content of the collection tube was discarded by pipetting.

After the first wash with 350 μ l of RW1 buffer, the sample was treated for 15 minutes with DNase I in order to eliminate DNA contamination. For every spin column, an aliquot of 10 μ l of DNase I was thawed on ice and then gently mixed with 70 μ l of buffer RDD. The mix was added directly to the spin column membrane for 15 minutes. The DNase treatment was followed by another wash with 350 μ l of buffer RW1 and 500 μ l of buffer RPE. After the final wash with 500 μ l of 80% ethanol, the spin column was dried by centrifugation for 5 minutes, with the lid open. 16 – 18 μ l of RNase-free water for the final elution was added directly to the column membrane and left for at least 5 minutes before centrifuging the spin column for 1 minute at full speed.

Samples of RNA for time 0 and 1 hour extracted from 0.5 ml aliquots were sent for RNA sequencing (RNA-seq) to the Exeter University Sequencing Service.

2.3 RNA extractions from time 1 hour (TRIzol™ Reagent)

This method, developed in 1987 (**Chacon-Cortes and Griffiths, 2014**), employs organic solvents: guanidinium thiocyanate, phenol, chloroform, isopropanol (**Ali et al., 2017**).

Table 4. Reagents used for RNA extractions with TRIzol™.

Reagent	Function in RNA extraction from <i>E. coli</i> cells
TRIzol™ (mixture of phenol, thiocyanic acid, guanidine, ammonium thiocyanate) (Thermo Fisher Scientific, b)	Phenol denatures RNases (Chacon-Cortes and Griffiths, 2014) Guanidine compounds are chaotropic agents: they denature the proteins associated with RNA (MacDonald et al., 1987) by interfering with their hydrogen bonds (Salvi et al., 2005). They contribute to the cell lysis (Ali et al., 2017).
Chloroform	Drives separation of the solid phase from the liquid phase containing RNA (Chacon-Cortes and Griffiths, 2014)
Isopropanol	Used to precipitate RNA from solution (Ali et al., 2017)

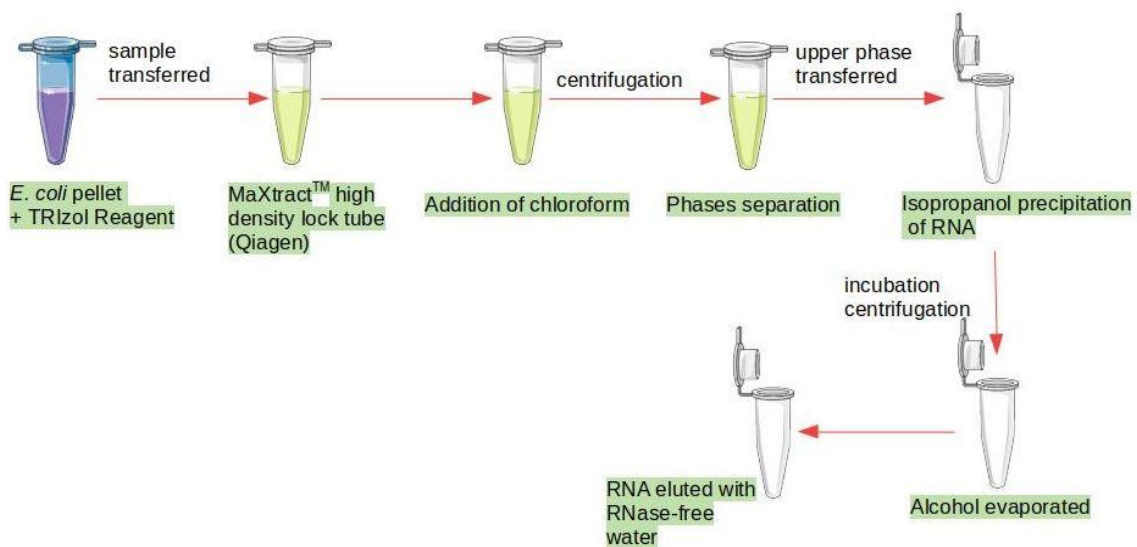
i. Samples preparation

RNA was extracted from pellets of 1 ml and 50 ml aliquots of the diluted overnight culture re-grown for 1 hour. The aliquots were spun down for 1 minute at full speed. The supernatant was carefully pipetted out. The pellet was re-suspended in 750 µl of TRIzol. The vortex was set to continuous mode and the tubes were

vortexed a few times for 1-2 seconds at a time. They were spun briefly for a few seconds in a centrifuge to assess if all the pellet was re-suspended.

ii. Extraction protocol

Figure 16: Workflow for RNA extractions from *E. coli* samples at time 1 hour, TRIzol Reagent (cartoons downloaded from *Smart Servier Medical Art* <https://smart.servier.com/>)



All centrifugation steps were performed in an Eppendorf 5810R centrifuge set at 4°C for the whole procedure.

Bacterial pellet re-suspended in TRIzol, was transferred into 1.5 ml MaXtract™ high density phase lock tubes, which were previously briefly centrifuged to bring the oil to the bottom. 150 µl of molecular-grade chloroform were added, the tubes were shaken by hand for a few seconds and left to incubate for 3 minutes. After that time, they were spun down for 5 minutes at 10000 rcf, 4°C. The result of centrifugation was separation of the content in three distinct phases: clear upper aqueous containing nucleic acids, middle white oil, and lower red phenol - chloroform. The upper phase, containing RNA separated from DNA and proteins (Ali *et al.*, 2017), from each tube was carefully transferred with a pipette into a new 1.5 ml LoBind Safe Lock Eppendorf™ tube. Then, 375 µl of 99.5%

isopropanol (Acros Organics) was added, in order to precipitate the RNA (**Ali et al., 2017**). The tubes were inverted 6 to 10 times and left to incubate for 10 minutes at room temperature. After, they were centrifuged for 10 minutes at 12000 ×g, at 4°C, which caused RNA to sediment at the bottom (white jelly pellet). The supernatant was quickly discarded by tipping the tubes and the pellet was re-suspend in 75% of ethanol. The tubes were briefly vortexed and centrifuged for 5 minutes at 10000 rcf, 4°C. Most of the supernatant containing ethanol was discarded by tipping the tubes. A brief centrifuge allowed the remaining ethanol to flow to the bottom and could be discarded by careful pipetting. The opened tubes were left at room temperature to allow remaining ethanol to evaporate. This process caused the pellet to change colour from milky (ethanol still present) to clear. Care was taken to keep the tubes' content safe from contamination, for example from dust. The final step was re-suspending RNA in RNase-free water: 12 µl for the 1 ml aliquot sample, 22µl for the 50 ml aliquot sample. The volumes of RNase-free water for re-suspension were an arbitrary choice, rather than a recommendation of a manufacturer's protocol.

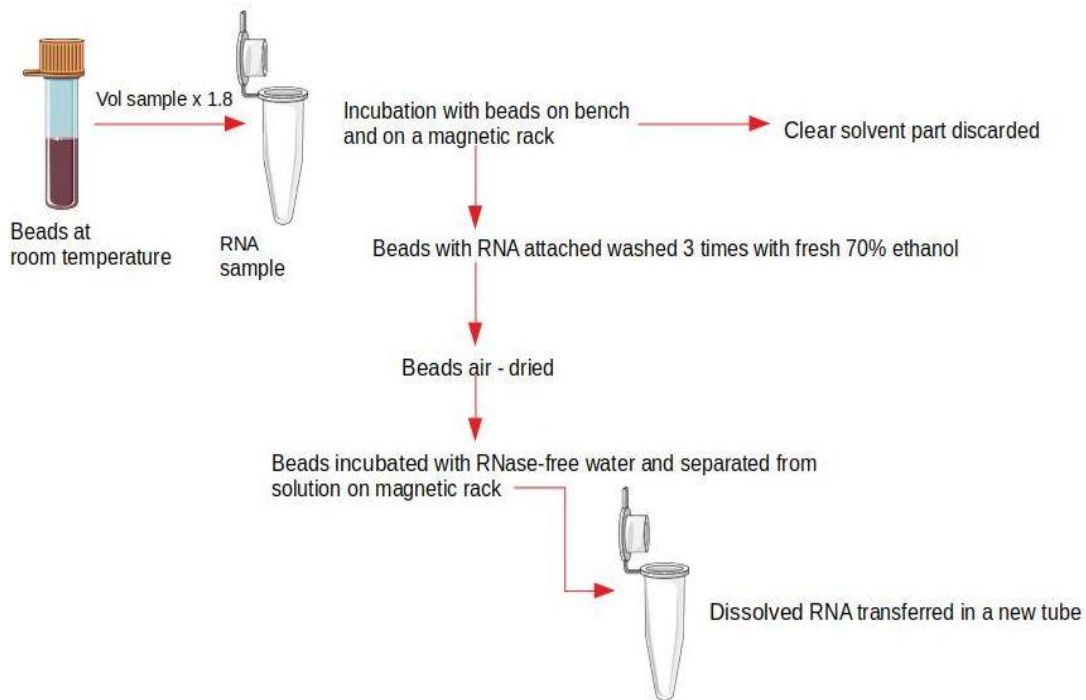
2.4 RNA purification

The Exeter Sequencing Service requires RNA samples to be free from DNA, wash buffers or other contaminants (**Exeter Sequencing Service, 2015**)

The method of choice for RNA purification was magnetic beads technology: Agencourt® RNA Clean XP® beads (**Beckman Coulter**), [Figure 17](#). The exact composition of the product is confidential. The SDS indicates that the beads mixture contains a low concentration of sodium azide and has a pH between 8.0 and 8.4. The method does not involve any centrifugation steps (**Berensmeier, 2006**). It relies on selective binding of the nucleic acid by coated magnetic particles, which can be collected and immobilised by applying a magnet to the outside of the reaction tube (**Oberacker et al., 2019**). The elution step allows to separate the nucleic acid from the beads, which are again collected when the tube is placed on the magnetic rack (**Berensmeier, 2006**).

Similarly to RNA extractions, the procedure was performed on a clean bench and by the Bunsen burner. 70% ethanol used for the wash step, was made with RNase-free water in an RNase-free Falcon tube. As recommended in by the manufacturer, the diluted ethanol was always no older than one week to ensure its correct concentration. Only filter pipette tips and reaction tubes certified as RNase-free were used.

Figure 17: Workflow for RNA purification with Agencourt® RNA Clean XP® beads; (cartoons downloaded from *Smart Servier Medical Art* <https://smart.servier.com/>). This method relies on selective binding of the nucleic acid by coated magnetic particles, which can be collected and immobilised by applying a magnet to the outside of the reaction tube (**Oberacker et al., 2019**).



An aliquot of beads was first brought to room temperature and mixed by vortexing to re-suspend the sedimented beads. The volume of the beads for a given reaction was calculated according to manufacturer's instructions: (volume of the sample \times 1.8). After 5-minute incubation of the sample with beads on the bench and a further 5 minutes on a magnetic rack, the clear solvent portion of the sample was very carefully aspirated with a pipette. Subsequently, beads with RNA attached were washed 3 times with 500 μ l of 70% ethanol. A few-second centrifuge step allowed to bring the remaining drops of ethanol to the bottom of the reaction tube, so they could be aspirated with a small-size pipette. The air-drying step had variable time length compared to producer's instructions. The beads were considered ethanol-free when from shiny they would become matt. The final elution with 26 μ l RNase-free water was the last step. The sample was

left to incubate with water for 5 minutes prior to being placed on a magnetic stand. The solution containing RNA was aspirated and transferred into a clean RNase-free tube.

2.5 Assessment of RNA quantity and quality

After the extractions or purification with magnetic beads, aliquots of 2 μ l were immediately transferred to separate RNase-free tubes to use for assessment of RNA quantity and quality. The remaining RNA sample was frozen and stored at -80°C. If necessary, the sample was thawed on fresh ice later.

Nano Drop

Nano Drop 2000c Spectrophotometer (Thermo Scientific™) values were used as a rough guideline on sample's purity and concentration (in ng/ μ l). The option for RNA was selected in the software. The Nano Drop pedestal was first gently cleaned with a Kimwipe™ (Kimberly-Clark™). A blank sample of 2 μ l RNase-free water was tested to calibrate the instrument. RNA quality was given by the ratios of absorbance values of:

- 260 nm/280nm; indicates sample's quality and potential contamination with protein, considered ideal around the 2.0 value (**Qiagen, 2014; Matlock, 2015**)
- 230 nm/260nm; ideally should be around 2.0 – 2.2; lower ratio can indicate potential contamination from guanidine or phenol which are components of reagents used for RNA extractions (**Matlock, 2015**).

Qubit®

A Qubit® fluorometer and its associated RNA High Sensitivity or Broad Sensitivity reagents (Invitrogen), were used to assess an RNA sample concentration. As indicated by the manufacturer, an appropriate volume of the working solution was prepared, mixed in 0.5 ml PCR tubes with fluorescent standards for instrument's calibration, and with 2 μ l of each RNA sample. The tubes were left at room temperature for about 2 minutes before the measurement. The sample's concentration was given as μ g/ml, which equals to ng/ μ l.

Tape station

Agilent 2200 Tape station and its associated High Sensitivity or Broad Sensitivity reagents (Agilent Technologies, Germany) were used to assess RNA quality, after purification with Agencourt® RNA Clean XP® beads and control of sample's concentration with Qubit. If the Qubit reading indicated a sample concentration close to the upper range of a given Tape sensitivity (10 ng/µl for High Sensitivity and 500 ng/µl for Broad Range), the sample was diluted with RNase-free water. Following manufacturer's instructions, the reagents and the tape were brought to room temperature before use. The RNA sample was mixed with an appropriate buffer aliquoted with reverse pipetting technique. After 1 minute of vortex mixing and heating for 3 minutes at 72°C in a PCR thermocycler, the tubes were loaded into the Tape Station together with a corresponding tape and pipette tips. The option of prokaryotic RNA was selected from the software and the lanes for each sample were described. The RNA quality was given as the equivalent of an RNA Integrity Number (RIN^e) (**Jahn et al., 2008**) ranging from 0 (lowest quality) to 10 (highest quality), assigned to a sample by 2100 Bioanalyzer system (Agilent Technologies, Germany) (**Agilent Technologies**).

2.6 Effect of Agencourt® RNA Clean XP® beads

We compared Nano Drop readings before and after the lag phase samples were cleaned with magnetic beads. It is worth noting that, according to the Nano Drop manufacturer, a sample concentration below 10 ng/µl can result in unreliable 260/280 values (**Matlock, 2015**).

The process of purification improved the 260/230 ratio, which could indicate its role in eliminating residues of guanidine present in Qiagen kits buffers. In some cases, the Nano Drop ratio 260/280 was lower after the purification, which may indicate partial degradation of the sample (Table 5).

Nano Drop data suggested that the purification process with magnetic beads, caused some loss of the RNA sample.

Table 5: Examples of Nano Drop values before and after clean up with Agencourt® RNA Clean XP® beads.

RNA sample ID	Concentration (ng/µl)		Nano Drop 260/230 ratio		Nano Drop 260/280 ratio		RIN
	Before beads	After beads	Before beads	After beads	Before beads	After beads	
11/04/17	3.8	2.8	0.05	0.97	1.54	1.40	6.4
13/04/17	4.8	2.6	0.25	1.17	1.77	1.55	6.8
09/05/17	6.7	4.3	0.14	0.60	1.42	1.37	8.0
Time 1 hour	Before beads	After beads	Before beads	After beads	Before beads	After beads	
14/03/17	16.1	10.7	0.12	1.40	1.95	2.01	7.2
23/03/17	10.9	10.1	0.59	1.11	2.18	1.91	8.2
30/03/17	21.2	6.2	0.49	2.34	1.96	2.38	8.3

2.7 RNA extractions from persisters and VBNCs (Qiagen kits)

The procedure of RNA extraction from phenotypically tolerant *E. coli* cells, was based on the methods described by **Keren et al. (2004b)**: an overnight stationary phase culture was diluted in a fresh medium, allowed to reach the exponential phase, and challenged with ampicillin. Bacteria were sedimented by centrifugation prior to RNA extraction. The authors used a strain of *E. coli* with mutations in the *hipA* gene ([Table 6](#)) which have been associated with proportions

of persisters a few orders of magnitude higher compared to the wild type (Balaban *et al.*, 2004). According to Keren *et al.*, (2004a), the numbers of *E. coli* persisters after challenge with ampicillin remains on a constant level during the lag phase, and increases during the exponential phase: a two-fold increase for the wild type *E. coli* HM21, between the time 3 and 4 hours of culture growth (Figure 7). Orman and Brynildsen (2013a) determined that the exponential phase of *E. coli* MG1655 contained more VBNC cells than persisters.

These findings published in the literature suggested that the exponential phase was appropriate for RNA extractions from persisters and VBNCs.

Table 6. Comparison of conditions of bacterial growth prior to RNA extraction, between the work described by Keren and colleagues (2004b) and the work described in this dissertation.

Conditions of RNA extraction from persisters and VBNC cells	Keren <i>et al.</i> , (2004b) 'Specialized persister cells and the mechanism of multidrug tolerance in <i>Escherichia coli</i> '	Experiments for this dissertation
<i>E. coli</i> strain	HM22 (genotype AT984 <i>zde-264::Tn10 hipA7</i>)	BW25113 (wt)
Overnight cultures	Diluted aliquot of bacteria from an 8% dimethyl sulfoxide (DMSO) stock frozen at -80°C, grown at 37°C, 250 rpm, 16 – 20 hours.	One colony from an LB agar streak plate (prepared from a glycerol stock frozen at -80°C) in 200 ml of LB in a 500 ml flask, grown at 37°C, 200 rpm, 17 hours.

Conditions of RNA extraction from persisters and VBNC cells	Keren et al., (2004b) 'Specialized persister cells and the mechanism of multidrug tolerance in <i>Escherichia coli</i>'	Experiments for this dissertation
Growth medium	Lysogeny Broth supplemented with 75 µg/ml of diaminopimelic acid.	Lysogeny Broth with 0.5 g/L of NaCl, no additional supplementation of the medium.
Time point at which ampicillin was added	Early exponential phase: 2.5 hours after the overnight culture was diluted 1:1000 in 600 ml of fresh LB (3 L flask).	Late exponential phase: 4 hours after the overnight culture was diluted 1:1000 in 100 ml of fresh LB (250 ml conical flask).
Ampicillin concentration	50 µg/ml, treatment for 3 hours.	125 µg/ml, ampicillin stock dissolved in water and no older than 7 days; treatment for 3 hours.
Temperature	Cells unlysed by ampicillin sedimented on ice, centrifuged at 4°C	All centrifugation steps of the procedure performed at 4°C.

For the protocol described here, the RNA was extracted in the same way as for untreated samples (Section 2.2 and [Figure 15](#)): addition of the RNA Protect®Bacterial Reagent, lysosyme and Tris EDTA incubation, transfer of the sample into a silica spin column with subsequent wash steps, DNase I treatment, and final elution with RNase-free water.

The Bunsen burner was not used here, but particular care was taken not to contaminate the sample with RNases from the researcher's lab coat, hair, skin, saliva, or from dust in the environment. All the centrifugation steps were performed at 4°C for the whole procedure to further protect the integrity of RNA. The ampicillin concentration used was 125 µg/ml, which corresponds to 25xMIC for the *E. coli* BW25113 strain used in the laboratory (**Bamford et al., 2017**), [Figure 14](#). Ampicillin was added directly to the aliquots of culture re-growing for 4 hours at 37°C ([Figure 13](#)); the samples were then incubated for 3 hours with aeration on a shaker set to 200 rpm.

RNA was extracted from 0.5 ml and 50 ml aliquots. For 0.5 ml aliquot of treated bacteria, 1 ml of RNeasy Protect® Reagent was added directly for 5 to 10 minutes. 50 ml aliquots in Falcon tubes, were first spun down for 5 minutes at full speed (3220 rcf). The pellet was then re-suspended by pipetting in 0.5 ml of spent LB (Section 2.7.1). From this point the aliquot was treated in the same way as 0.5 ml sample. After incubation of the samples with the RNeasy Protect® Reagent, the unlysed cells were pelleted by vigorous centrifugation: a range of speeds was tested for different samples, from 1699 rcf to 18000 rcf, and time between 5 and 10 minutes.

For intermediate centrifugation steps Qiagen protocol recommendation was at least 8000 ×g. Here, the speed of 10000 rcf was used throughout the protocol.

2.7.1 Spent LB preparation

Spent LB is the consumed medium of liquid culture; it was prepared as described by **Bamford et al. (2017)** who investigated the VBNC phenotype in *E. coli*, using a single-cell approach of the Mother Machine. The spent medium was prepared by spinning down at room temperature 50 ml aliquots of the overnight culture at 3220 rcf in an Eppendorf 5810R centrifuge for 10 minutes. The supernatant was filtered twice with a 0.22 µm filter syringe (Minisart™ Sartorius) in order to separate the liquid phase from bacteria cells and solid debris.

Chapter 3: Results and Discussion

In their 2017 paper “The *E. coli* molecular phenotype under different growth conditions” **Caglar and colleagues** described how gene expression of bacterial cells was shaped by the phase of growth. The researchers found that gene expression was similar between cells in exponential or in stationary phase, independently on the additional stress like carbon or magnesium starvation. Gene expression analysis of *E. coli* BW25113 (wild type) in different stages of culture growth was part of the research described by **Smith et al., (2018)** in the paper “The Culture Environment Influences Both Gene Regulation and Phenotypic Heterogeneity in *Escherichia coli*”. The RNA extractions at times 0 and 1 hour of culture growth, as described in the previous chapter, completed the RNA extractions performed by the research group. The gene expression analysis included over 4300 genes (Figures 20 - 21), which have been listed in the Table 1 of Supplementary Materials published with the paper.

3.1 Approximate numbers of *E. coli* cells used for the experiments

Values of CFU/ml and OD₆₀₀ were only collected for time points 0, 1, 4, 17 hours, used for this work (Figure 18 A-C); no data were taken for the intermediate time points. For times 0 and 1 hour, the OD₆₀₀ was between 0 and 0.1, for time 4 hours (no ampicillin treatment) it ranged between 0.6 and 0.76. In order to obtain the OD for 17 hours, the value measured (1:10 dilution in fresh LB), was multiplied by 10. The values for time 17 hours varied between 3.6 and 5.7. **Sezonov et al., (2007)** indicate OD₆₀₀ value of 7 as the point of *E. coli* K-12 culture saturation in LB medium.

For time 17 hours, the CFU/ml are within 10⁹ order of magnitude, while for time 0, it is 10⁶ order of magnitude. This is consistent with a 1:1000 dilution of the overnight culture. A similar trend is present in Figure 7 from the paper by **Keren et al., (2004a)**: circles mark CFU/ml versus time for untreated *E. coli* cells, where

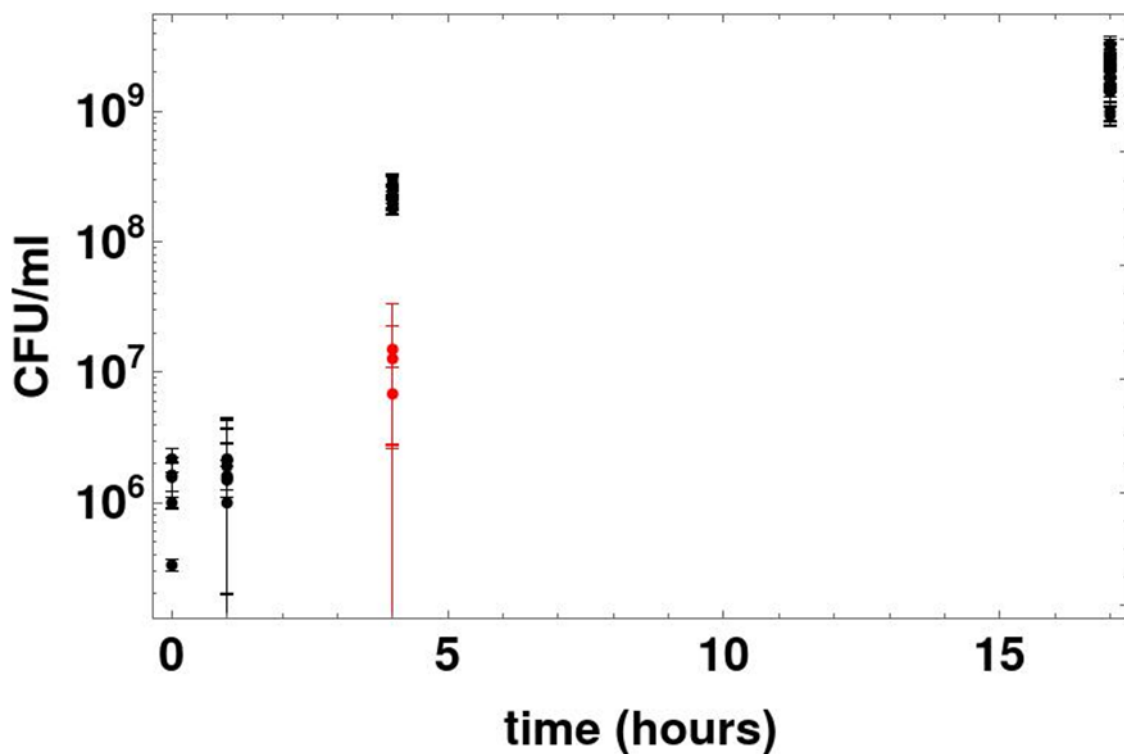
a dilution 1:1000 of the stationary-phase culture lowers the CFU/ml count from 10^{10} to 10^7 .

Figure 18:

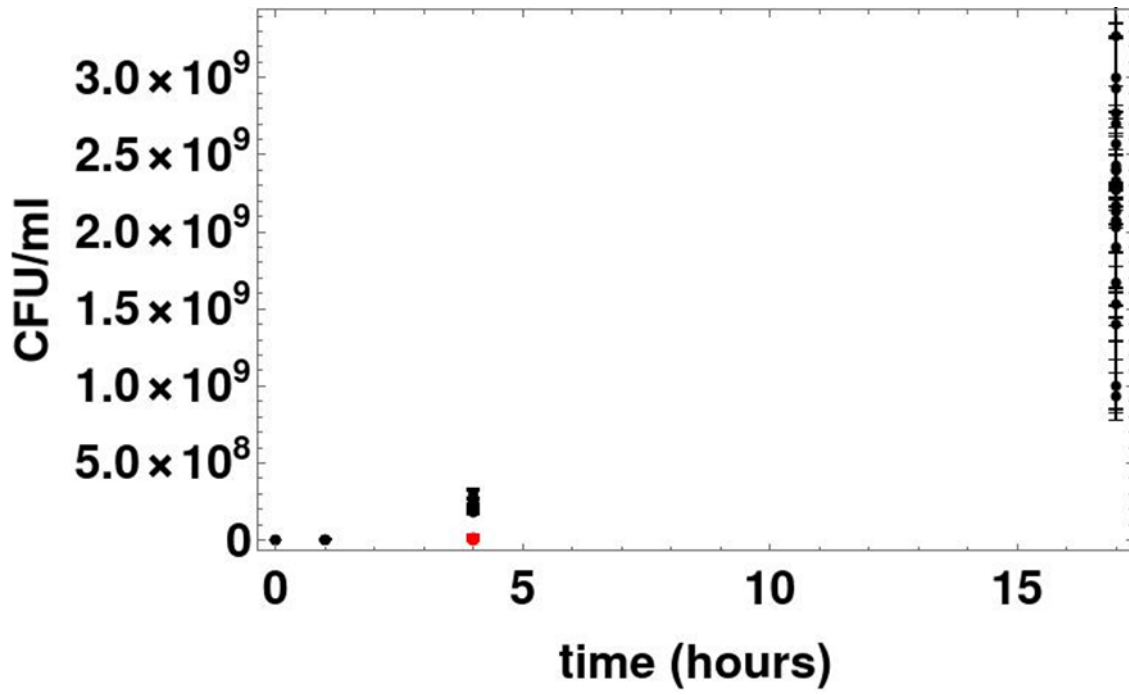
A) and B) The average number of CFU/ml counted for the time points 0, 1, 4, 17 hours. Red dots are values for time point 4 hours after 3 hours of ampicillin treatment ($25\times$ MIC). A) and B) present the same data in a different scale to show the wide spread of the error bars (standard deviation).

C) Values of CFU/ml and OD_{600} for time points 0, 1, 4, 17 hours, measured for the purpose of this work, using methods described in Sections 2.1i and 2.1ii. Error bars are standard deviation.

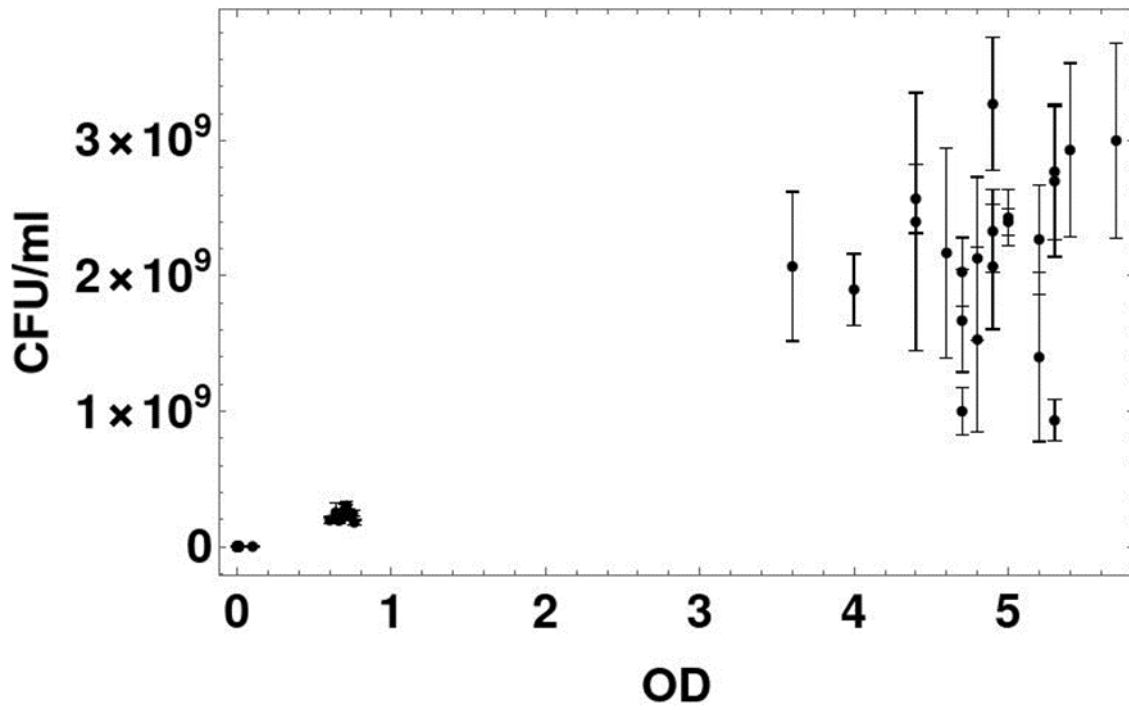
A)



B)



C)



3.2 Results of RNA extractions from untreated *E. coli* in lag phase

As shown in [Figure 3](#), time 0 is the earliest stage of the lag phase, when cells begin awakening from stationary phase while being provided fresh growth medium (**Madar et al., 2013**). Small numbers of cells and a lack of biomass pose challenges in the study of the lag phase of growth (**Shultz and Kishony, 2013**). Research by **Jöers and Tenson (2016)** on *E. coli* showed that not all cells recover from the stationary phase at the same rate, which is another example of heterogeneity of bacterial populations, even without additional environmental challenges like presence of antibiotics.

In their review, **Brauner et al., (2016)** describe bacterial tolerance to antibiotics during the lag phase due to a transient growth arrest when cells are recovering in the new environment after the stationary phase. These facts, combined with bacterial ability to evolve extended lag phase over a few generations under antibiotics pressure (**Brauner et al., 2016**), make this phase of growth an important contributor to bacterial tolerance. This phase is also important from the point of view of food safety. For example, **Gill et al., (2001)** determined that *E. coli* bacteria in pork meat can survive in the lag phase at low temperatures, and resume growth when the environmental temperature is raised.

The results presented in this chapter illustrate some of the complexities of the lag phase in *E. coli* grown in rich LB medium.

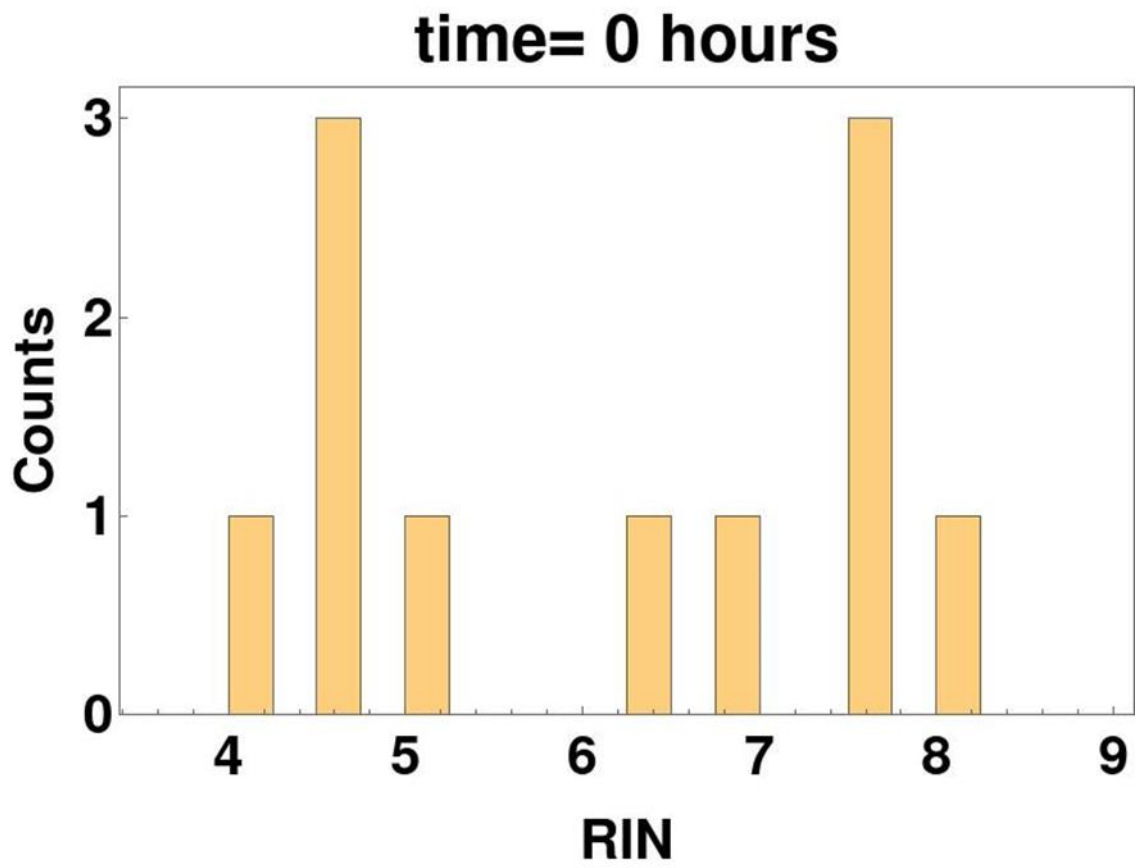
3.2.1 RNA quality and quantity (lag phase, no treatment)

Table 1S (Supplementary Materials) and [Figures 19 – 20](#), show results of RNA extractions performed from samples in the lag phase, where the quality and the quantity assessment produced values within the lower range of the instruments' detection. The CFU/ml plating method ([Figure 13](#)) suggested that the numbers of cells used for the extractions were similar for both time points 0 and 1 hour (Section 3.1). As shown in [Figures 19A and 19C](#), extraction of good-quality RNA

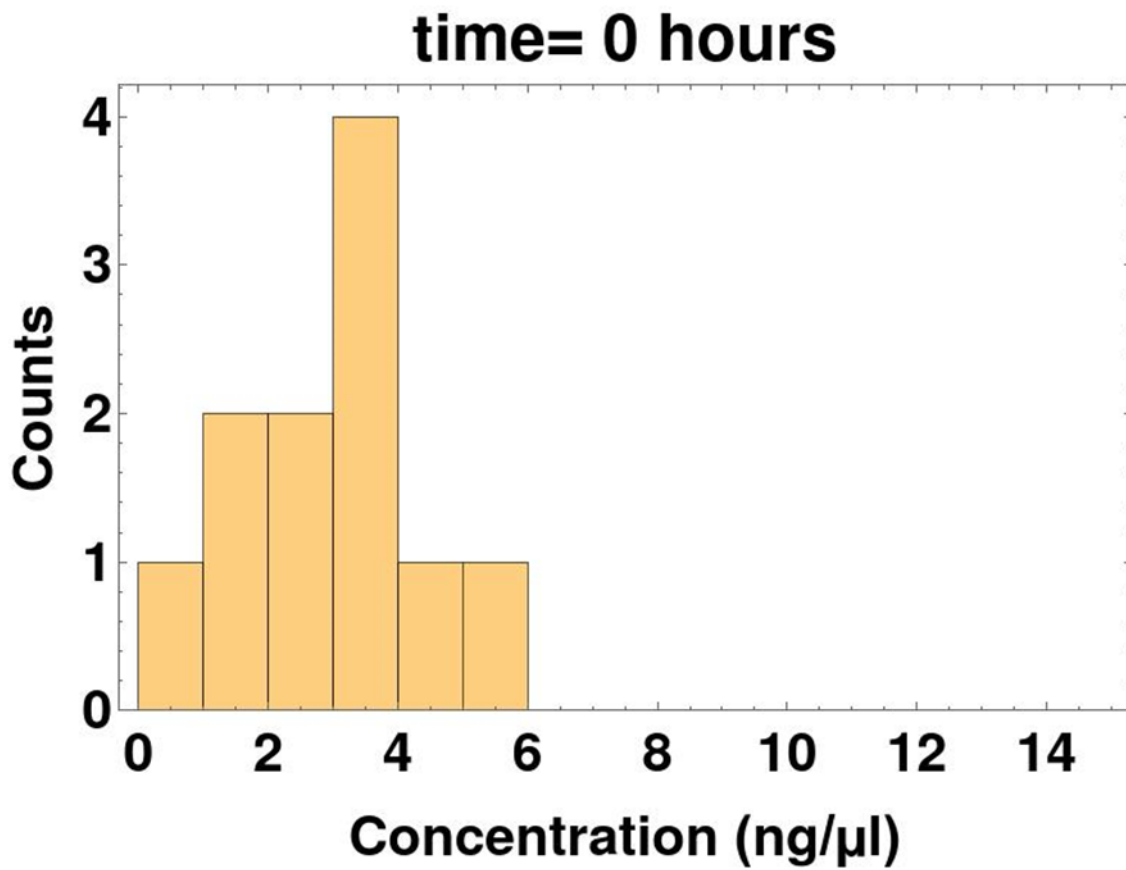
from samples at time point 0 hours proved challenging: out of 11 samples, only 4 of them approached the RIN value of 8 (red dots). Figure 20 A and C show RIN scores for time 1 hour: out of 13 samples, 9 had the RIN value of 8 or more (red dots). As shown in Figures 19 B and 20 B, as well as in Table 1S (Supplementary Material), the quantity of extracted RNA from times 0 and 1 hour fluctuated between the different attempts for each of the time points. Variability of CFU/ml of the time point 17 hours (Figure 18 A-C) indicates a varying quantity of the starting material for the extractions. Pipetting inaccuracy during the extractions, resulting in loss of sample, could also have contributed to the fluctuations of extracted RNA. For time 0 hours, RNA concentration (in ng/ μ l) varied between 1 and 6, while for time 1 hour it varied between 3.2 and 14.3. Figures 19 C and 20 C show a plot of RIN versus RNA concentration, where each point represents one experiment. The available data points are insufficient to define a clear relationship between the RNA quality and quantity.

Figure 19: Results of RNA extractions from time point 0 hours, for untreated *E. coli* cells, using Qiagen RNeasy kit. Extraction was performed immediately after the initial 1:1000 dilution of the overnight culture. The graphs show the data reported in Table 1S (Supplementary Materials), no technical replicates, each point is a single experiment. **A)** Histogram showing RIN distribution between the different experiments; **B)** Histogram showing concentration distribution between the different experiments; **C)** Plot of RIN versus RNA concentration: red dots show data points with the highest RIN values.

A)



B)



C)

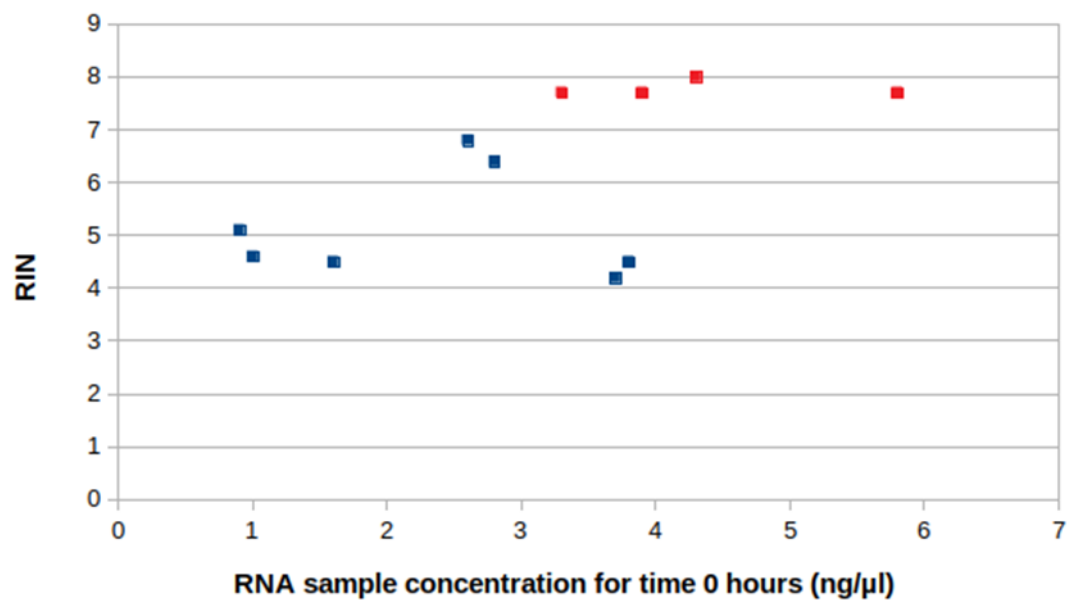
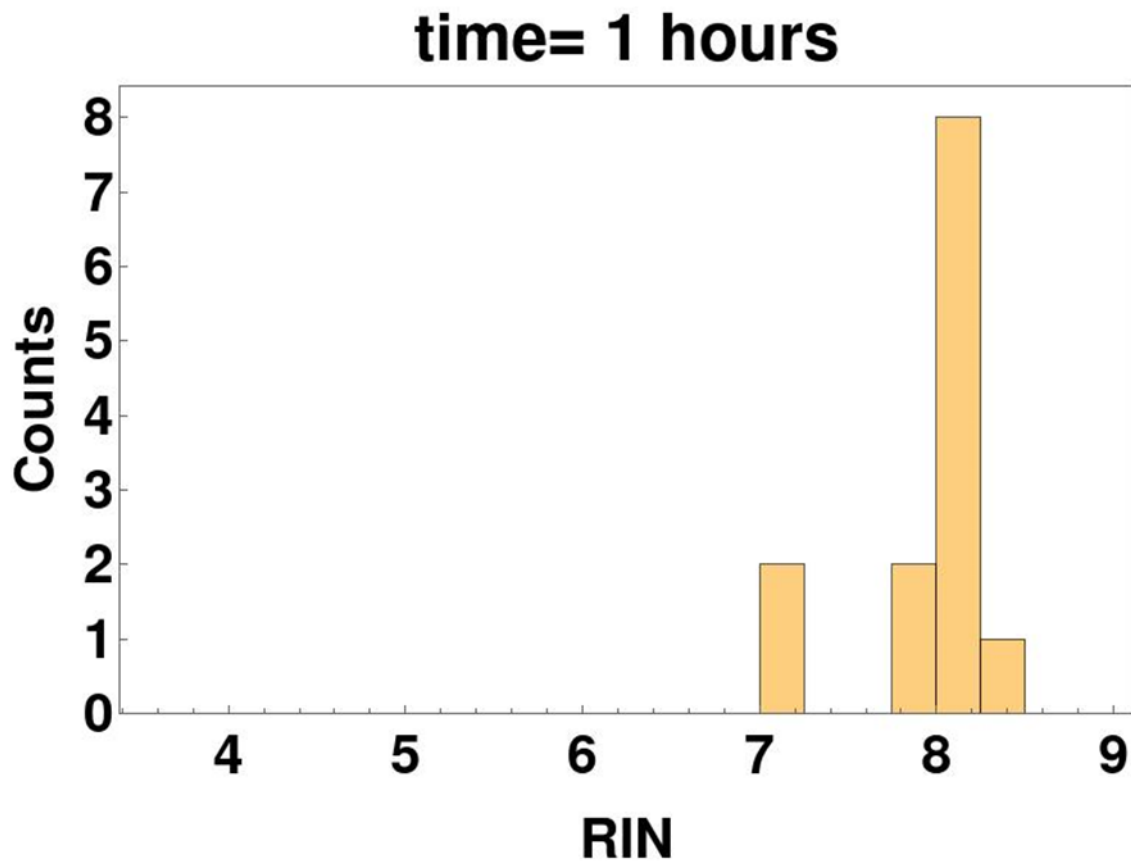
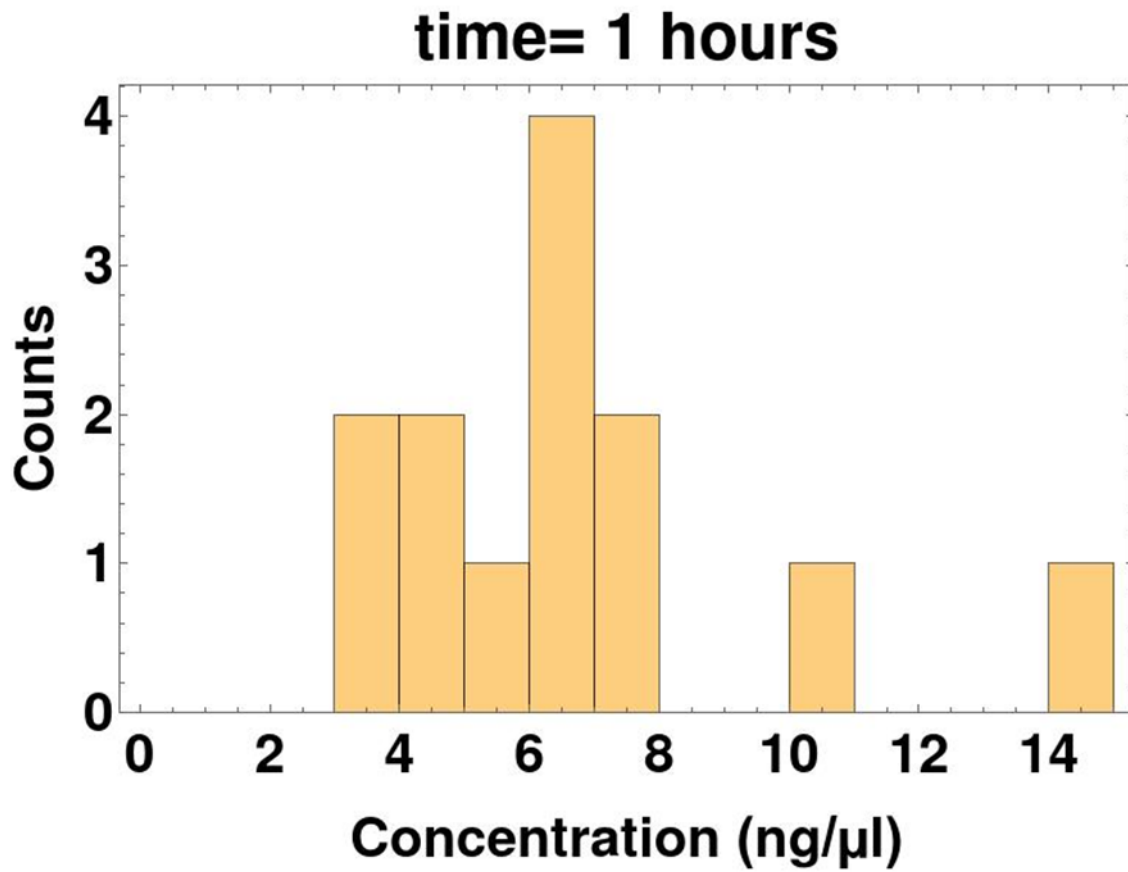


Figure 20: Results of RNA extractions from time point 1 hours, for untreated *E. coli* cells, using Qiagen RNeasy kit. The graphs show the data reported in Table 1S (Supplementary Materials), no data for technical replicates, each point is a single experiment. **A)** Histogram showing RIN distribution between the different experiments; **B)** Histogram showing concentration distribution between the different experiments; **C)** Plot of RIN versus RNA concentration: red dots show data points with the highest RIN values.

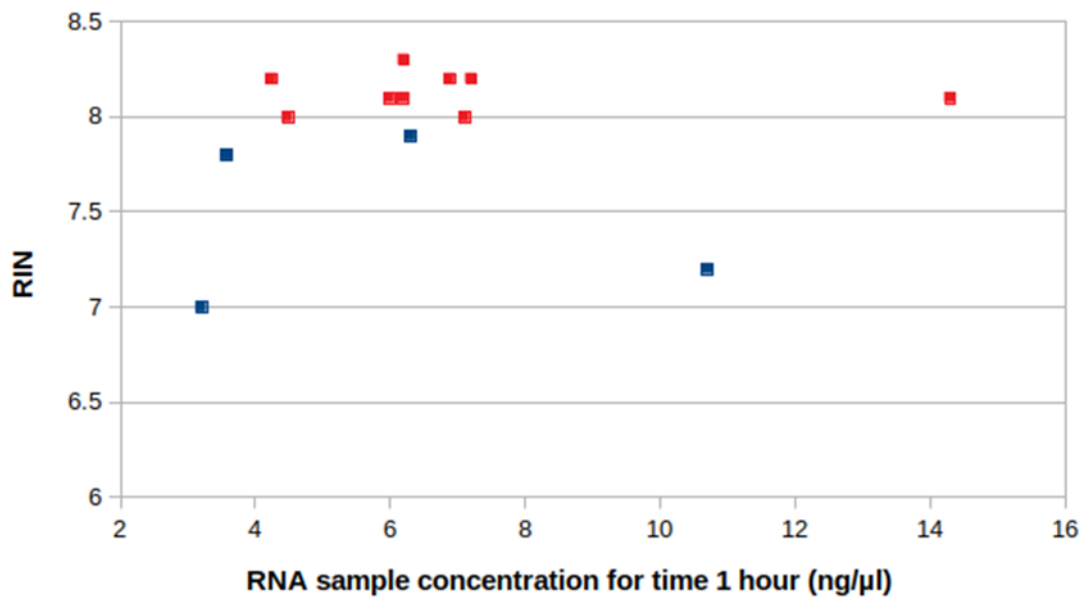
A)



B)

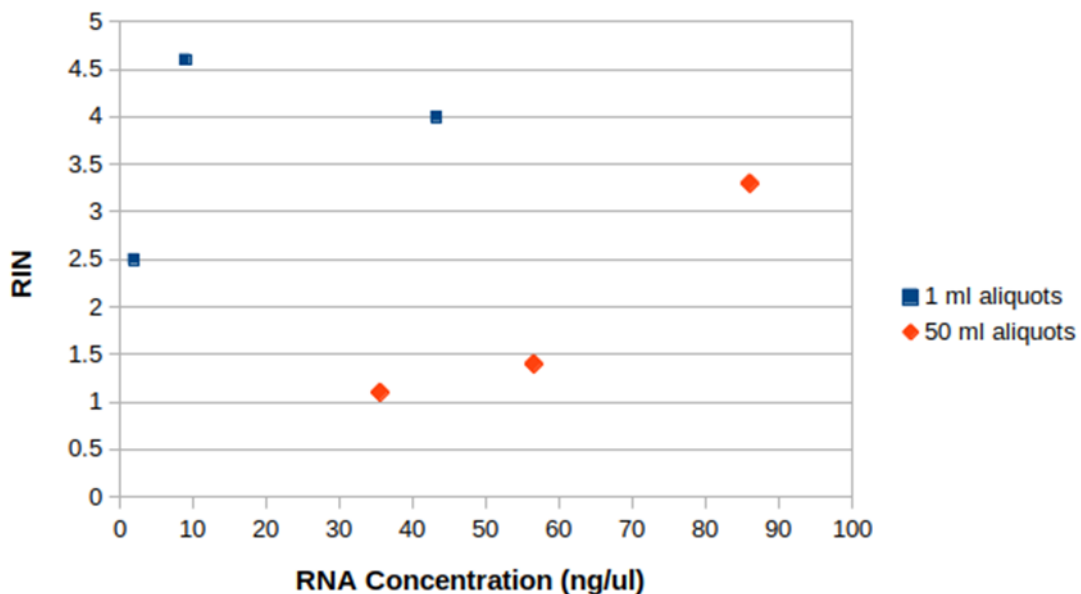


C)



RNA extractions using the TRIzol™ Reagent from time point 1 hour, as shown in Figure 14, were only attempted for the total of 6 times for two different aliquots of the re-growing culture (Figure 16, Table 2S), in order to test the method. Also in this case, the extractions were not successful, since the RIN scores obtained were low. This could be due to the sample contamination during the extraction, for example when the liquid phase containing RNA was collected by aspiration after chloroform treatment (Section 2.3).

Figure 21: Graph showing RIN values versus RNA concentration, for extractions from time point 1 hour, using TRIzol™ Reagent. Every point represents a single experiment.



Independently from the method used, in order to preserve the RNA integrity, a lot of attention was paid to limit as much as possible potential contamination of the reagents and the plastics with environmental RNases, for example from skin and dust (**Ambion**). The bench was always cleaned with 70% ethanol and RNase Erase spray (MP Biomedicals, UK). When possible, the bacteria handling for OD₆₀₀ measurement and 1:1000 dilution were performed on a separate bench. The equipment, like vortex, pipettes, or centrifuge, was cleaned with ethanol and

RNase Erase spray prior to the extractions. Gloves were also cleaned or changed if they touched any surfaces presenting potential contamination with RNases, like skin, lab coat, or dust (**Ambion**). Reaction tubes and filter pipette tips were all certified RNase-free. Ethanol absolute was always diluted with RNA-free water to the required concentration.

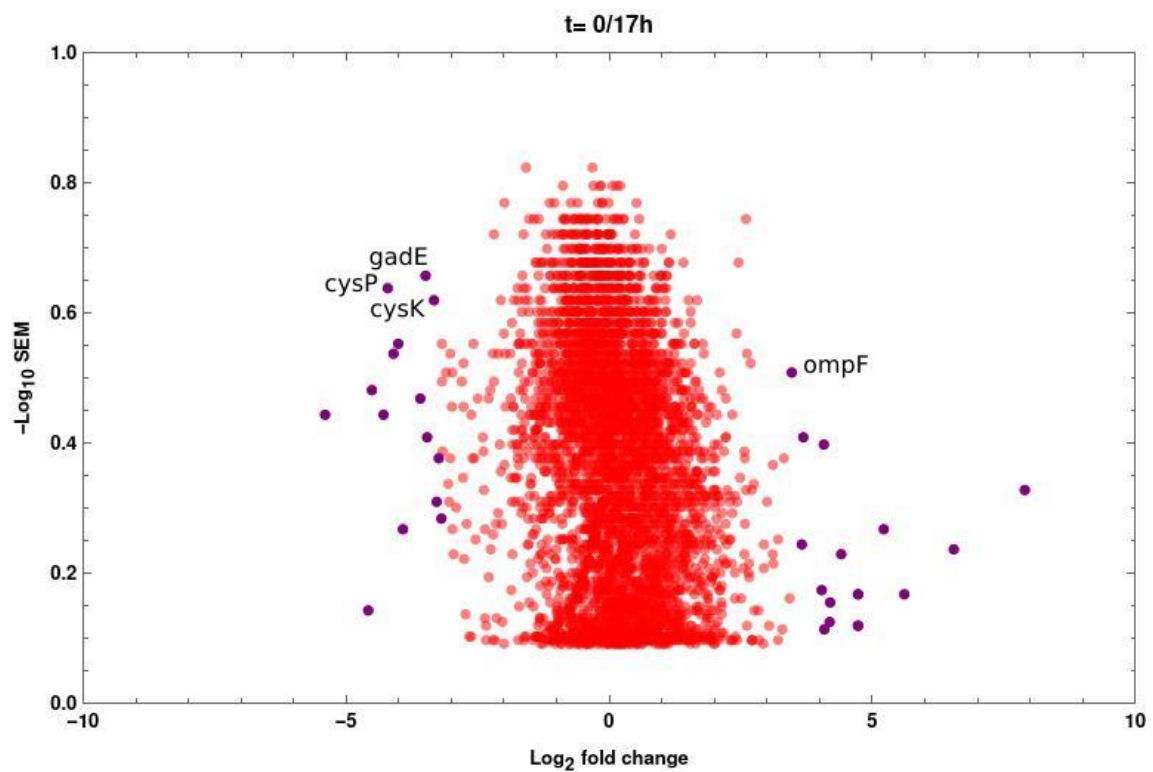
3.2.2 The overall gene expression pattern at times 0 ad 1 hour of culture growth

All gene expression experiments begin with extraction of RNA from the cells of interest (**RNA-seqlopedia**). For the purpose of this work, only untreated RNA samples were sequenced. They were extracted using Qiagen kits ([Figure 15](#)). The first step of the protocol was treatment of the bacteria aliquot with RNAprotect® Bacteria Reagent, which preserves the RNA. This “freezing” of RNA in time is important, since the cells undergo genetic reprogramming during the lag phase of growth (**Madar et al., 2013**).

At t=0h, 73% of the analysed genes fall in the interval of -1 and +1 Log₂-fold change compared to the values for time 17 hours ([Figure 22](#)). The distribution close to the value of zero is consistent with the way the time zero of the growth culture is prepared ([Figure 13](#)): an aliquot of the stationary phase (17 hours) inoculated into a fresh medium. The logical consequence is for the transcript reads normalized against time 17 hours and presented as logarithms, to be close to 0, since the log of 1 = 0. This suggests a minimal or no change to the expression of most genes.

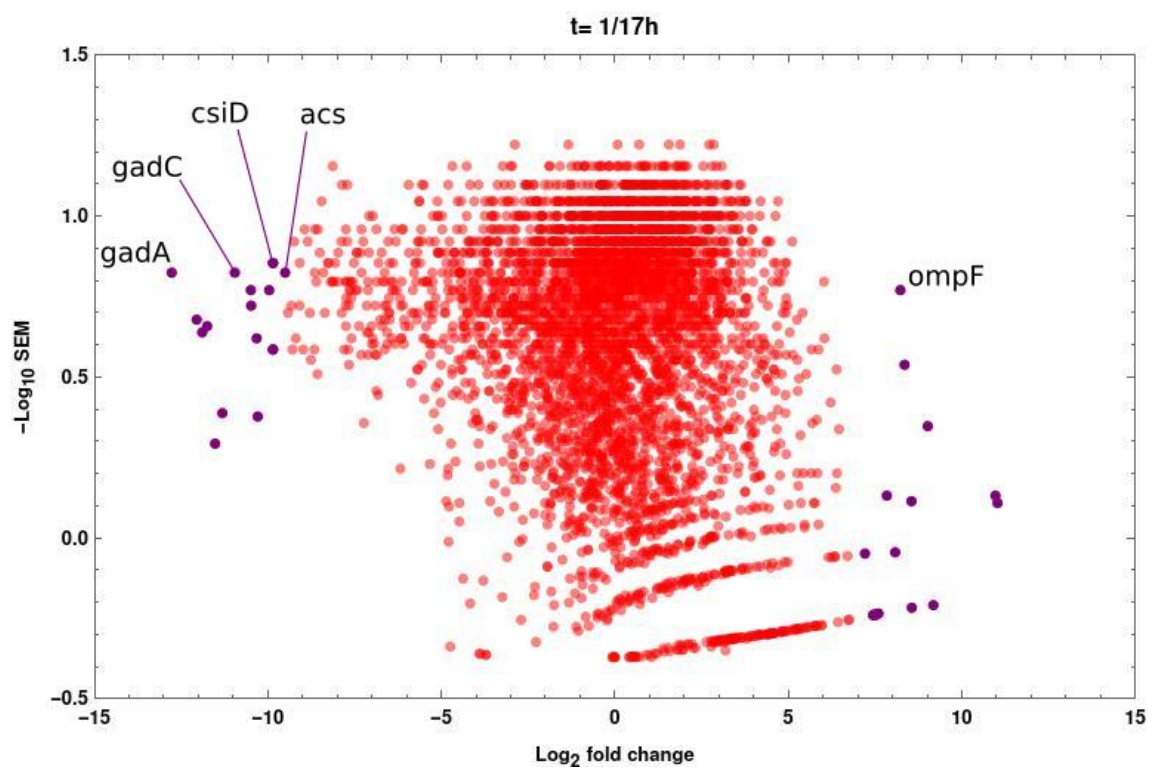
Figure 22: Frequency distribution of Log₂-fold change against the -Log₁₀SEM (Standard Error of Mean) for the time point 0 hour of culture growth of *E. coli* BW25113 (wild type) without treatment; data published in Table 1 of Supplementary Materials of **Smith et al., (2018)**. Purple dots are the 15 most up- and down-regulated genes for this time point, listed in Tables 7 and 9. Transcripts with high -Log₁₀SEM values (small statistical error) have been labelled for the

most down-regulated genes (purple dots to the left) and the most up-regulated genes (purple dots to the right).



At t=1h, less than a half of the genes fall in the interval between -1 and +1 of the Log₂-fold change (Figure 23). This indicates the genetic re-programming of bacteria growing in the fresh medium with aeration for one hour, which **Rolfe et al., (2012)** observed in their gene expression investigation of *Salmonella enterica* serovar Typhimurium during the lag phase.

Figure 23: Frequency distribution of Log_2 -fold changes against the $-\text{Log}_{10}\text{SEM}$ (Standard Error of Mean) for the time point 1 hour of *E. coli* BW25113 (wild type) culture growth without treatment; data published in Table 1 of Supplementary Materials of **Smith et al., (2018)**. Purple dots are the 15 most up- and down-regulated genes for this time point, listed in Tables 8 and 10. Some values of Log_2 -fold change for the most up- and down-regulated genes are not visible as separate purple points on the plot. Transcripts with high $-\text{Log}_{10}\text{SEM}$ values (small statistical error) have been labelled for the most down-regulated genes (purple dots to the left) and the most up-regulated genes (purple dots to the right).



Another way to look at the gene expression data, is to list some of the most up- and down-regulated genes, compared to the baseline time point 17 hours. From the dataset of over 4000 genes listed in the Table 1 Supplementary Materials of **Smith et al., (2018)**, 15 of the most up-regulated, and 15 of the most down-regulated genes are listed in Tables 7 – 10 for time points 0 and 1 hour. The number of genes selected for the tables was an arbitrary choice, aimed at illustrating some of the genetic reprogramming of *E. coli* at the two time points of interest.

3.3 The most up-regulated genes

Table 7 List of the 15 most up-regulated genes for the time point 0 hours of *E. coli* BW25113 (wt) grown in nutrient LB medium, and the corresponding values for time point 1 hour. The data have been extracted from Table 1 Supplementary Materials of **Smith et al., (2018)**. The numerical values are normalised to transcript reads obtained from aliquots of RNA extracted at time point 17 hours. Protein names and Gene Ontology Biological Processes annotation, come from the UniProt database (<https://www.uniprot.org/>), accessed the 25 of August 2019. The entries marked in yellow are amongst the 15 most up-regulated genes common both for time point 0 hour and 1 hour (Table 8).

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours SEM	Mean log2 fold change t=1/17 hours SEM	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 3618	cspA	14.67	9.67	7.9 (SEM:	11.03	Cold shock protein CspA

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours SEM	Mean log2 fold change t=1/17 hours SEM	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
				0.47) SEM	(SEM: 0.78)	GO: response to cold
Gene 1004	cspG	2.33	1.45	6.55 (SEM: 0.58)	7.84 (SEM: 0.74)	Cold shock-like protein CspG GO: response to cold
Gene 1403	<i>ynaE</i>	0.67	0.33	5.61 (SEM: 0.68)	5.07 (SEM: 0.93)	Uncharacterized protein YnaE GO: response to cold
Gene 2432	<i>lpxP</i>	15.33	4.33	5.22 (SEM: 0.54)	4.2 (SEM: 0.26)	Lipid A biosynthesis palmitoleoyltransferase GO: lipid A biosynthetic process; lipopolysaccharide biosynthetic process; response to cold
Gene 1586	<i>cspI</i>	1	0.58	4.73 (SEM: 0.68)	4.27 (SEM: 0.8)	Cold shock-like protein CspI GO: response to cold

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours SEM	Mean log2 fold change t=1/17 hours SEM	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 1593	<i>cspF</i>	0	0	4.73 (SEM: 0.76)	9.18 (SEM: 1.62)	Cold shock-like protein CspF
Gene 1592	<i>cspB</i>	5.33	1.33	4.41 (SEM: 0.59)	5.82 (SEM: 0.43)	Cold shock-like protein CspB GO: response to cold
Gene 1577	<i>ydfK</i>	1.67	1.2	4.2 (SEM: 0.7)	3.12 (SEM: 0.66)	Cold shock protein YdfK GO: response to cold
Gene 3663	<i>rhsJ</i> (synonym <i>ym</i> <i>yibJ</i>)	0	0	4.19 (SEM: 0.75)	7.49 (SEM: 1.73)	Putative uncharacterized protein YibJ
Gene 1250	<i>tpr</i>	0.33	0.33	4.09 (SEM: 0.77)	8.08 (SEM: 1.11)	Protamine-like protein.
Gene 273	<i>yagF</i>	13.33	5.04	4.08	0.14	D-xylonate dehydratase YagF

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours SEM	Mean log2 fold change t=1/17 hours SEM	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
				(SEM: 0.4)	(SEM: 0.33)	GO: aldonic acid catabolic process
Gene 1762	ydiY	1	0.58	4.04 (SEM: 0.67)	10.97 (SEM: 0.74)	Uncharacterized protein YdiY
Gene 457	<i>ybaZ</i> (synonym <i>ati</i>)	11.67	2.03	3.69 (SEM: 0.39)	0.08 (SEM: 0.32)	DNA base-flipping protein GO: DNA repair
Gene 2219	<i>fruB</i>	19.33	8.88	3.66 (SEM: 0.57)	4.6 (SEM: 0.27)	Multiphosphoryl transfer protein GO: fructose import
Gene 941	ompF	84.67	22.28	3.47 (SEM: 0.31)	8.23 (SEM: 0.17)	Outer membrane porin F GO: drug transmembrane transport; ion transmembrane transport

Table 8. List of the 15 most up-regulated genes for the time point 1 hour of *E. coli* BW25113 (wt) grown in nutrient LB medium, and the corresponding values for time point 0 hour. The data have been extracted from Table 1 Supplementary Materials of **Smith et al., (2018)**. The numerical values are normalised to transcript reads obtained from aliquots of RNA extracted at time point 17 hours. Protein names and Gene Ontology Biological Processes annotation, come from the UniProt database (<https://www.uniprot.org/>), accessed the 25 of August 2019. The entries marked in yellow are amongst the 15 most up-regulated genes common both for time point 1 hour and 0 hour (Table 7).

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours SEM	Mean log2 fold change t=1/17 hours SEM	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 3618	cspA	14.67	9.67	7.9 (SEM: 0.47)	11.03 (SEM: 0.78)	Cold shock protein CspA GO: response to cold
Gene 1762	ydiY	1	0.58	4.04 (SEM: 0.67)	10.97 (SEM: 0.74)	Uncharacterized protein YdiY
Gene 1593	cspF	0	0	4.73 (SEM: 0.76)	9.18 (SEM: 1.62)	Cold shock-like protein CspF GO: Cold shock-like protein
Gene 4211	<i>cadB</i>	3.67	0.33	2 (SEM: 0.77)	9.02 (SEM: 0.45)	Probable cadaverine/lysine antiporter GO: L-lysine transmembrane transport; cadaverine transport; cellular stress response to acidic pH

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours SEM	Mean log2 fold change t=1/17 hours SEM	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 2036	<i>yoeG</i>	0	0	2.93 (SEM: 0.81)	8.56 (SEM: 1.65)	No entries in the UniProt database
Gene 2060	<i>yoeI</i>	1	0.58	1.07 (SEM: 0.79)	8.55 (SEM: 0.77)	Uncharacterized protein Yoel
Gene 4210	<i>cadA</i>	11.67	1.67	1.7 (SEM: 0.63)	8.35 (SEM: 0.29)	Inducible lysine decarboxylase GO: role in pH homeostasis; catalytic activity in lysine processing
Gene 941	<i>ompF</i>	84.67	22.28	3.47 (SEM: 0.31)	8.23 (SEM: 0.17)	Outer membrane porin F GO: drug transmembrane transport; ion transmembrane transport
Gene 1250	<i>tpr</i>	0.33	0.33	4.09 (SEM: 0.77)	8.08 (SEM: 1.11)	Protamine-like protein
Gene 1004	<i>cspG</i>	2.33	1.45	6.55 (SEM: 0.58)	7.84 (SEM: 0.74)	Cold shock-like protein CspG GO: Response to cold.
Gene 4287	<i>yjfZ</i>	0	0	0.96 (SEM: 0.68)	7.6 (SEM: 1.72)	Uncharacterized protein YjfZ
Gene 1174	<i>ycgX</i>	0	0	0.45 (SEM: 0.53)	7.51 (SEM: 1.74)	Uncharacterized protein YcgX

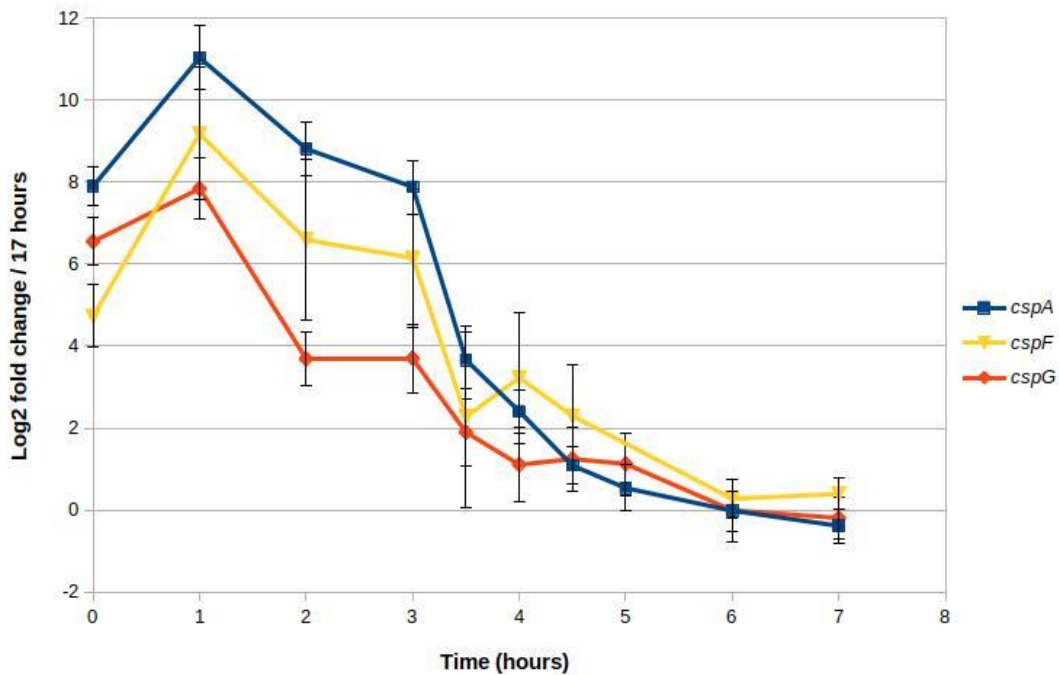
Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours SEM	Mean log2 fold change t=1/17 hours SEM	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 3663	<i>rhsJ</i> (synonym <i>yibJ</i>)	0	0	4.19 (SEM: 0.75)	7.49 (SEM: 1.73)	Putative uncharacterized protein YibJ
Gene 3028	<i>yghG</i>	0.33	0.33	0.09 (SEM: 0.52)	7.21 (SEM: 1.12)	Lipoprotein YghG; Part of secretion II system GO: cellular response to DNA damage stimulus
Gene 1003	<i>cspH</i> (synonym <i>cspD</i>)	0	0	3.21 (SEM: 0.8)	6.72 (SEM: 1.8)	Cold shock-like protein CspD GO: negative regulation of DNA replication; response to starvation

3.3.1 Response to cold shock

Among the 15 most up-regulated genes, half of them fall in the response to cold shock both for time 0 and time 1 hour, as shown in the [Tables 7 and 8](#). This is consistent with the fact that the aliquot of the overnight culture grown with aeration at 37°C, was diluted in fresh LB medium at room temperature ([Figure 12](#)). The changing levels of expression of genes *cspA*, *cspE*, *cspG* are shown on the graph in [Figure 24](#): they fall after time 1 hour, remain at a steady level at times 2 and 3 hours, then fall as the culture enters the exponential phase. This can be explained by the fact that the new culture is grown from time zero with aeration again at 37°C which causes the bacteria to down-regulate over time this cold

shock response to stress. The starting temperature of the fresh medium inoculated with the overnight bacterial culture, is not always stated in scientific literature (**Keren *et al.*, (2004 a)**, **Sezonov *et al.*, (2007)**; **Kragh *et al.*, 2018**). To the best of my knowledge, in the literature referenced for the purpose of this dissertation, only **Conter (2003)** clearly states that the fresh LB medium was pre-warmed before inoculation with an aliquot of the overnight culture.

Figure 24: Graph showing Log₂ fold-change for three genes involved in cold response in *E. coli* BW2113 (wild type). Data come from the Supplementary Materials published with the paper by **Smith *et al.*, (2018)**. Error bars are SEM.



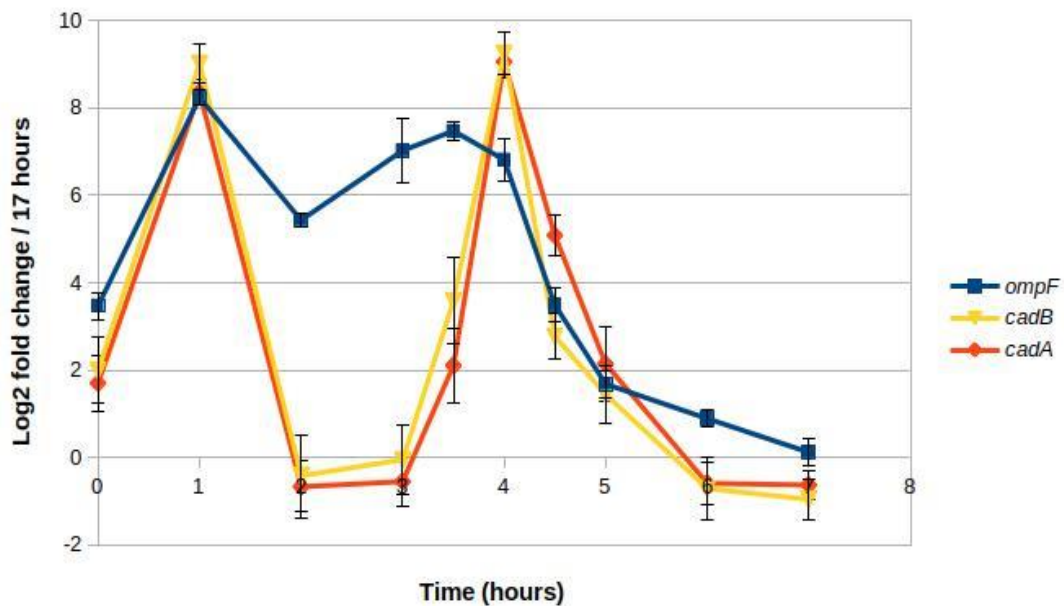
3.3.2 Resistance to mildly acidic conditions

Another interesting observation of the up-regulated genes comes from [Figure 25](#). At time points 1 and 4 hours the expression values of genes *cadA* and *cadB* reach the maximum levels: respectively 70 times higher than the baseline value at 17 hours, and 81 times higher than the baseline 17 hours. The *cad* genes encode a lysine/cadaverine antiporter located in the inner membrane, and the cytoplasmic lysine decarboxylase (**Kanje and Houry, 2013**). Decarboxylation of the amino acid lysine into cadaverine, allows *E. coli* cells to survive in a mildly acidic environment, within the pH range of 4 - 5. Presence of this acid resistance system is consistent with the observation of **Smith et al., (2018)**, published in the Supplementary Materials (Figure S4), that the pH of the LB medium drops during the lag phase to reach the lowest value of 6.2 at time point 4 hours, after which it increases steadily to about 7 until the time 8 hours.

The same graph shows the expression pattern of the gene *ompF*. The protein it encodes, belongs to the family of β -barrel-shaped outer membrane proteins present in all Gram-negative bacteria (**Rollauer et al., 2015**). OmpF location allows bacteria to move sugars, antibiotics, amino acids, ions, and to transduce signals across the membrane (**Koebnik et al., 2000**). OmpF expression is regulated by environmental factors, like the pH of the medium: **Nikaido (2003)**, reports that pH of 5.2 results in down-regulation of *ompF*. Mild acidic conditions reported by **Smith et al., (2018)** do not have this effect on this gene in the lag phase. During the lag phase, *ompF* level of expression increases about 27 times from time 0 to 1 hour, when it reaches the maximum level, then it falls approximately 7 times between 1 and 2 hours ([Figure 25](#)). Low osmolarity of the growth medium has been reported in scientific literature as an environmental condition driving increased expression of the OmpF porin (**Nikaido, 2003**). The LB medium used for our experiments was not supplemented with sugars, and only contained 0.5g/L of NaCl, which corresponds to 0.0085M. **Baldwin and Kubitschek (1984)** reported the 0.23 M of NaCl as optimal for *E. coli* growth (Section 1.2). Low osmolarity of LB medium used for our experiments, rather than

its pH, could potentially contribute to the up regulation of *ompF* in at time point 1 hour.

Figure 25: Graph showing Log2 fold-change for *ompF*, *cadA*, *cadB* genes in *E. coli* BW2113 (wild type). Data come from the Supplementary Materials published with the paper by **Smith et al., (2018)**. Error bars are SEM.



3.4 The most down-regulated genes

Similarly to the data shown in the previous two tables, Tables 9 and 10 list the 15 most down-regulated genes for the times 0 and 1 hour. Unlike in the case of the most up-regulated genes, there is no overlap between the data in the two tables, except for the gene *ymdF*. At time zero, *gadE* is among the 15 genes, while the other genes from this family are strongly down-regulated at time 1 hour.

Table 9. List of the 15 most down-regulated genes for the time point 0 hour of *E. coli* BW25113 (wt) grown in nutrient LB medium, and the corresponding values for time point 1 hour. The data have been extracted from Table 1 Supplementary Materials of **Smith et al., (2018)**. The numerical values are normalised to transcript reads obtained from aliquots of RNA extracted at time point 17 hours. Protein names and Gene Ontology Biological Processes annotation, come from the UniProt database (<https://www.uniprot.org/>), accessed the 25 of August 2019. The entries marked in yellow are amongst the 15 most up-regulated genes common both for time point 1 hour and 0 hour (Table 10).

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	Mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 2806	<i>cysD</i>	2177.33	403.26	-5.4 (SEM: 0.36)	-8.27 (SEM: 0.14)	Sulfate adenylyltransferase subunit 2 GO: Hydrogen sulfite biosynthetic process
Gene 67	<i>sgrT</i>	71.33	9.74	-4.58 (SEM: 0.72)	-4.56 (SEM: 0.35)	Putative inhibitor of glucose uptake transporter SgrT; Promotes recovery from glucose-phosphate stress

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	Mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
						GO: Negative regulation of glucose import
Gene 4411	<i>iraD</i>	807.67	127.22	-4.51 (SEM: 0.33)	-7.87 (SEM: 0.2)	Anti-adaptor protein IraD Promotes RpoS stability during oxidative stress; GO: Cellular response to DNA damage stimulus
Gene 2817	<i>cysH</i>	2103	310.95	-4.29 (SEM: 0.36)	-6.97 (SEM: 0.1)	Phosphoadenosine phosphosulfate reductase GO: Hydrogen sulfite biosynthetic process; Sulfur compound metabolic process

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	Mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 2479	<i>cysP</i>	2887.33	530.49	-4.21 (SEM: 0.23)	-8.4 (SEM: 0.15)	Thiosulfate-binding protein; Part of ABC complex CysAWTP GO: sulfate/thiosulfate import
Gene 1312	<i>yciW</i>	1855.67	334.26	-4.1 (SEM: 0.29)	-4.08 (SEM: 0.12)	Uncharacterized protein YciW
Gene 2818	<i>cysI</i>	5087.33	984.96	-4.01 (SEM: 0.28)	-7.74 (SEM: 0.16)	Sulfite reductase [NADPH] hemoprotein beta-component; part of a complex catalyzing sulfite to sulfide GO: Cysteine biosynthetic process; Sulfate assimilation

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	Mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 3696	<i>waaU</i>	112.33	19.7	-3.92 (SEM: 0.54)	0.13 (SEM: 0.25)	Lipopolysaccharide 1,2-N-acetylglucosaminetransferase GO: lipopolysaccharide core region biosynthetic process
Gene 2477	<i>cysW</i>	911.33	193.29	-3.59 (SEM: 0.34)	-7.68 (SEM: 0.26)	Sulfate transport system permease protein CysW; component of the ABC transporter complex CysAWTP responsible for sulfate/thiosulfate import
Gene 3571	<i>gadE</i>	10856.67	1953.55	-3.49 (SEM: 0.22)	-8.36 (SEM: 0.2)	Transcriptional regulator GadE; regulates genes involved in acid resistance

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	Mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
						GO: regulation of transcription
Gene 2805	<i>cysN</i>	1844.67	310.27	-3.46 (SEM: 0.39)	-6.86 (SEM: 0.16)	Sulfate adenylyltransferase subunit 1; involved in pathway of sulfate catalysis GO: Hydrogen sulfite biosynthetic process sulfur compound metabolic process
Gene 2468	<i>cysK</i>	12185.33	2022.78	-3.33 (SEM: 0.24)	-5.5 (SEM: 0.08)	Cysteine synthase A GO: Cellular amino acid biosynthetic process; Cysteine biosynthetic process from serine

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	Mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 1566	<i>yneM</i> (synonym <i>mgtS</i>)	166.67	26.72	-3.28 (SEM: 0.49)	-2.19 (SEM: 0.26)	Small protein MgtS; binds Mg ²⁺ transporter MgtA contributing to increased magnesium levels GO: cellular response to magnesium starvation
Gene 2804	<i>cysC</i>	162.33	28.53	-3.24 (SEM: 0.42)	-5.7 (SEM: 0.21)	Adenylyl-sulfate kinase GO: hydrogen sulfite biosynthesis; sulfate assimilation
Gene 1020	<i>ymdF</i>	41169	7782.04	-3.19 (SEM: 0.52)	-12.05 (SEM: 0.21)	Uncharacterized protein YmdF

Table 10. List of the 15 most down-regulated genes for the time point 1 hour of *E. coli* BW25113 (wt) grown in nutrient LB medium, and the corresponding values for time point 0 hour. The data have been extracted from Table 1 Supplementary Materials of **Smith et al., (2018)**. The numerical values are normalised to transcript reads obtained from aliquots of RNA extracted at time point 17 hours. Protein names and Gene Ontology Biological Processes annotation, come from the UniProt database (<https://www.uniprot.org/>), accessed the 25 of August 2019. The entries marked in yellow are amongst the 15 most up-regulated genes common both for time point 1 hour and 0 hour (Table 9).

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 3577	<i>gadA</i>	47382.67	8588.92	-1.21 (SEM: 0.25)	-12.77 (SEM: 0.15)	Glutamate decarboxylase alpha; components of the gad system which allows to regulate cell's internal pH when exposed to acidic conditions GO: glutamate metabolic process; intracellular pH elevation
Gene 1020	<i>ymdF</i>	41169	7782.04	-3.19	-12.05	Uncharacterized protein YmdF

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
				(SEM: 0.52)	(SEM: 0.21)	
Gene 965	<i>rmf</i>	66938	1523.97	-2.98 (SEM: 0.31)	-11.89 (SEM: 0.23)	Ribosome modulation factor GO: negative regulation of translation in response to stress
Gene 1525	<i>gadB</i>	34357	5642.31	-0.92 (SEM: 0.22)	-11.75 (SEM: 0.22)	Glutamate decarboxylase beta; part of the gad system GO: glutamate metabolic process; intracellular pH elevation
Gene 1280	<i>yjiG</i>	967.33	136.32	-2.71 (SEM: 0.53)	-11.52 (SEM: 0.51)	Uncharacterized protein YciG GO: bacterial-type flagellum-dependent swarming motility
Gene	<i>yjdN</i>	1329.67	226.99	-0.61	-11.31	Protein YjdN

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
4184				(SEM: 0.37)	(SEM: 0.41)	
Gene 1524	<i>gadC</i>	40228	6124.87	-1.54 (SEM: 0.24)	-10.95 (SEM: 0.15)	Probable glutamate/gamma-aminobutyrate antiporter; imports glutamate with simultaneous export of gamma-aminobutyric acid (GABA) produced by GadA and B. GO: amino acid transport; intracellular pH elevation
Gene 1220	<i>ychH</i>	5410.67	666.08	-1.32 (SEM: 0.41)	-10.49 (SEM: 0.17)	Uncharacterized protein YchH GO: cellular response to cadmium ion; cellular response to hydrogen peroxide; single-species biofilm

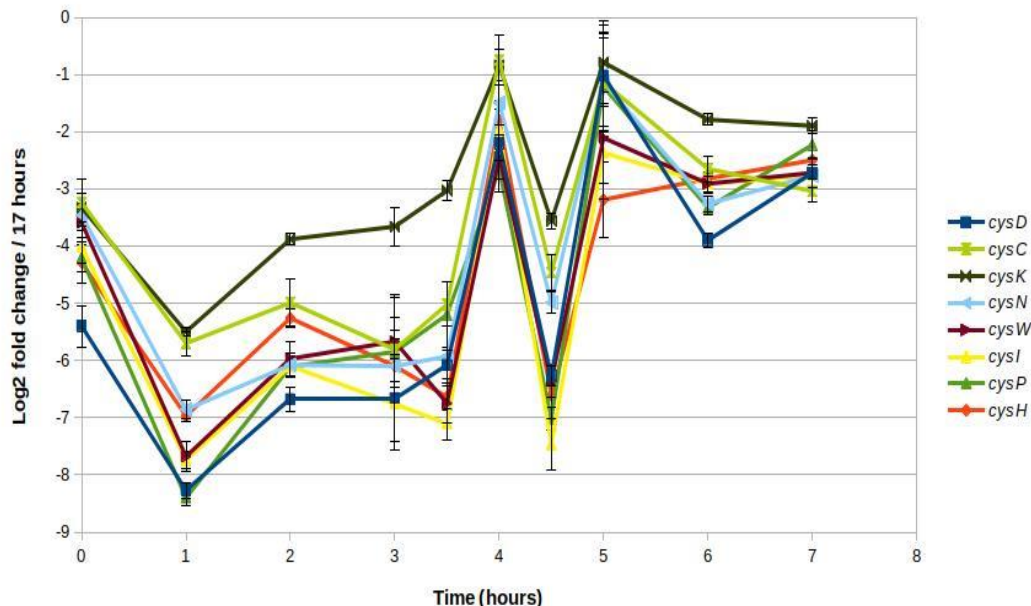
Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
						formation on inanimate substrate
Gene 909	<i>ycaC</i>	5296	866.8	-1.69 (SEM: 0.33)	-10.48 (SEM: 0.19)	Probable hydrolase YcaC
Gene 847	<i>bssR</i>	17748.33	2868.99	-1.12 (SEM: 0.26)	-10.32 (SEM: 0.24)	Biofilm regulator BssR GO: regulation of gene expression; regulation of single-species biofilm formation
Gene 984	<i>hyaA</i>	810.67	221.84	0.04 (SEM: 0.75)	-10.29 (SEM: 0.42)	Hydrogenase-1 small chain GO: anaerobic respiration; fermentation
Gene 1512	<i>sra</i>	8984	1192.81	-2.5 (SEM: 0.34)	-9.96 (SEM: 0.17)	Stationary-phase-induced ribosome-associated protein GO: translation

Gene ID	Gene name	Mean transcript reads at time 17 hours	SEM for the transcript reads at time 17 hours	Mean log2 fold change t=0/17 hours (SEM)	mean log2 fold change t=1/17 hours (SEM)	Encoded protein name and Gene Ontology (GO) Biological process for <i>E. coli</i> strain K12.
Gene 2713	<i>csiD</i>	5716	991.25	-0.46 (SEM: 0.48)	-9.85 (SEM: 0.14)	Glutarate 2-hydroxylase; involved in amino acid degradation GO: L-lysine catabolic process; response to carbon starvation
Gene 489	<i>glsA</i>	1260.67	241.88	0 (SEM: 0.33)	-9.85 (SEM: 0.26)	Glutaminase 1 GO: glutamine metabolic process; negative regulation of growth; response to acidic pH
Gene 4146	<i>acs</i>	3304.33	529.94	-0.02 (SEM: 0.43)	-9.5 (SEM: 0.15)	Acetyl-coenzyme A synthetase; involved in acetyl – CoA synthesis necessary for TCA cycle to generate cell's energy GO: acetyl-CoA biosynthetic process from acetate.

3.4.1 Sulphur metabolism

At time zero, the group of eight *cys* genes, all involved in sulphur metabolism stands out ([Table 9](#)). *cysD*, *cysC*, *cysN*, *cysH* are genes involved in the synthesis of hydrogen sulfite HSO_3^- ; proteins encoded by the genes *cysP* and *cysW* play a role in the import of thiosulfate $\text{S}_2\text{O}_3^{2-}$ in the cell; *cysI* and *cysK* are involved in the synthesis of cysteine (**Sekowska et al., 2000**). It is one of the two amino acids containing sulfur; its synthesis is linked to that of methionine, the other amino acid containing sulfur (**Carroll et al., 2005**). In the initiation of the mRNA translation ([Figure 8](#)), the AUG mRNA codon is bound by the initiator tRNA charged with methionine (**Solomon et al., 2009**), which makes the sulfur-containing amino acids indispensable for protein synthesis. As reported by **Sekowska et al., (2000)** in their review, sulphur metabolism is an energetically expensive series of reactions. As shown in [Figure 26](#), the level of expression of the *cys* genes initially falls between times zero and 1 hour, with the biggest fluctuations occurring between times 3.5 and 5 hours. A strong down-regulation of these genes at 1 hour suggests that the cells divert their resources to more crucial processes at this stage of culture growth.

Figure 22: Graph showing Log₂ fold-change for eight *cys* genes in *E. coli* BW2113 (wild type). Data come from the Supplementary Materials published with the paper by **Smith et al., (2018)**. Error bars are SEM.



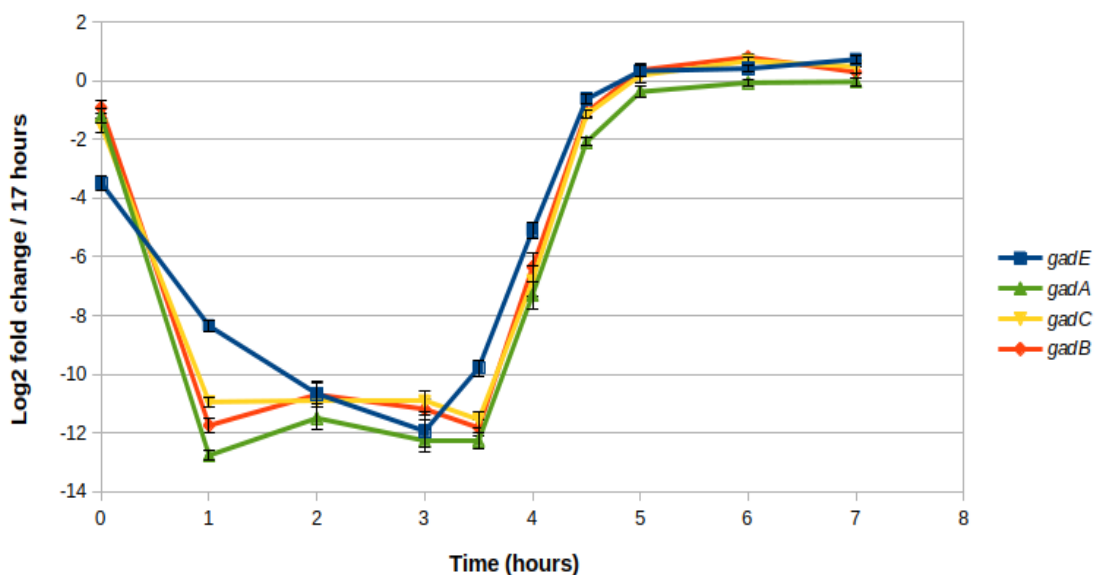
3.4.2 Resistance to strong acidic stress

Amongst the 15 most down-regulated genes for the time 1 hour, there are three *gad* genes. [Figure 27](#) also includes the *gadE*, which is amongst the most down-regulated genes at time zero. They encode the glutamate/GABA antiporter located in the inner membrane, as well as the cytoplasmic glutamate decarboxylase (**Kanjee and Houry, 2013**). This acid resistance system allows *E. coli* to maintain the internal pH close to the neutral level (**Bergholz et al., 2007**), when the environmental pH decreases to the values of 2 - 3 (**DeBiase et al., 1999**). It has been observed that the GAD system plays a protective role during extreme acid conditions of the stationary phase of growth (**DeBiase et al., 1999**) or allows pathogenic *E. coli* to survive acidic conditions of animal stomach (**Castanie-Cornet et al., 1999**). The down-regulation of the *gad* genes seems to be in contrast with the up-regulation of the *cad* genes mentioned in the paragraph 3.3.2. However, the *cad*-encoded acid resistance system is activated with mild

acid stress (up to the pH of 4), while the *gad*-encoded system is activated with pH values up to 2. The principle for both these systems is removal of a carbonyl group from an amino acid, with associated release of CO₂, and transport of the decarboxylation product across the inner membrane.

Bacteria constantly adapt to their fluctuating environment (Section 1.1); it is not surprising that more than one acid stress response system exists in the same strain, depending on the pH conditions of the growth medium (**Kanje and Houry, 2013**).

Figure 27: Graph showing Log₂ fold-change for four *gad* genes in *E. coli* BW2113 (wild type). Data come from the Supplementary Materials published with the paper by **Smith et al., (2018)**. Error bars are SEM.



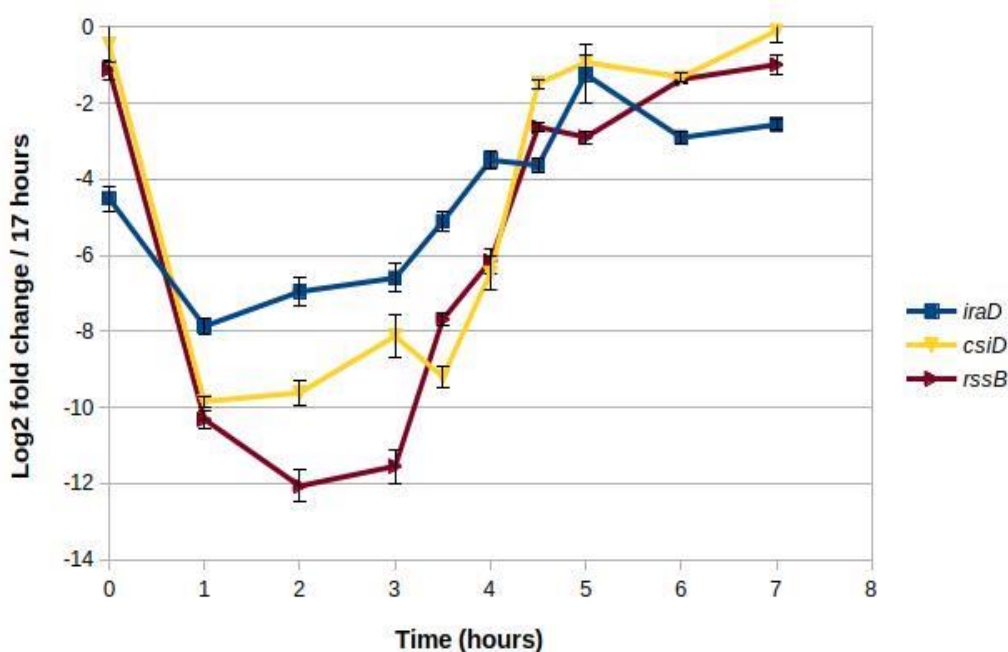
3.4.3 Genes associated with the sigma factor RpoS

Response to stress in *E. coli* often involves expression of a sigma factor RpoS (**Marschall et al., 1998**), which controls the expression of many genes, like *csiD*, by interaction with RNA polymerase (**Battesti et al., 2011**). In their review, **Battesti et al., 2011** state that the expression of RpoS is induced by conditions like unfavourable pH, or nutrient depletion in the stationary phase. *csiD* is a gene

induced by RpoS in response to carbon depletion in the medium during the stationary phase (**Marschall et al., 1998**). Results published by **Smith et al., (2018)** show that *csiD* is strongly down-regulated in the lag phase when the culture is supplied with fresh medium, and its levels start climbing during the exponential phase ([Figure 28](#)).

The activity of RpoS is strictly regulated; amongst the genes encoding regulatory proteins there are *iraD* and *rssB* (**Battesti et al., 2011**). The RssB protein associates with RpoS during the phases of cell growth, when the sigma factor is destined for degradation. IraD is one of the proteins which blocks the association with RssB and thus promotes the RpoS stability in the cell (**Battesti et al., 2011**). Therefore, it can be expected that both *rssB* and *iraD* genes would be strongly down-regulated early in the lag phase, when the cells are recovering from stress and preparing for division.

Figure 28: Graph showing Log₂ fold-change for two genes involved in RpoS regulation in *E. coli* BW2113 (wild type) and for the *csiD* gene which is regulated by RpoS. Data come from the Supplementary Materials published with the paper by **Smith et al., (2018)**. Error bars are SEM.



These examples of transcriptomic results illustrate that, even without any additional external pressures other than increasing cell density and changes to

the medium (nutrients depletion, altered pH) over time, bacterial cells constantly adjust their gene expression. It is a fine interplay between many genes which form regulatory pathways that transduce the signals from the external environment. The results described here prove that the changing conditions in a growth medium cause *E. coli* to adapt their gene expression.

3.5 RNA extractions from persisters and VBNC cells in exponential phase of culture growth

As detailed in Table 5, the method of isolation of tolerant sub-populations of *E. coli* adopted for this work, relies on prolonged treatment of *E. coli* cultures with a high concentration of ampicillin. The non-lysed cells are considered to be the tolerant sub-populations: Keren and colleagues (2004b) only mention persister cells, while Orman and Brynildsen (2013a) report an enrichment of cultures with VBNC cells. As mentioned in Section 2.7, attempts of isolating RNA from persisters and VBNC cells yielded inconsistent results in terms of RNA quantity, which seemed to increase over the course of subsequent extractions. This may have been caused by inefficient isolation of unlysed bacterial cells by centrifugation, or different quantities of starting material available for extractions, due to variability of ampicillin treatment efficiency. The attempts to isolate RNA from aliquots of 50 ml of bacteria proved challenging due to large quantity of lysed cells obstructing the Qiagen spin column. There was also a concern that the step of centrifuging the sample, and re suspending the thick pellet in spent LB could potentially introduce changes in bacterial transcriptome. The ampicillin-treated samples were not sequenced for the purpose of this dissertation, due to the inconsistencies of the extractions results and the RNA sequencing costs.

Persisters and VBNC cells have been recognised as threats to antibiotic efficacy (Section 1.6). This study concentrates on laboratory-adapted *E. coli* cultures, grown in a specific medium in controlled conditions, which differ significantly from the conditions within living organisms (Cambier *et al.*, 2014). However, a better understanding of the bacterial tolerance to antibiotics in the laboratory environment, is the first step towards potential solutions of the issue. As

discussed in the next chapter, the work presented here could potentially become a starting point for future attempts to isolate RNA from the tolerant sub populations of bacterial cultures growing in laboratory environment.

3.5.1 Use of spent LB

The aim of using the spent growth medium to re-suspend the pellet of 50 ml aliquots (Section 2.7), was to avoid providing the bacteria with a fresh source of nutrients during the incubation with the RNAprotect® Bacteria Reagent. **Bamford et al., (2017)** adopted spent LB in their microfluidic Mother Machine set up to investigate the VBNC phenotype.

However, as described in Table 2, the RNAprotect® Bacteria Reagent disrupts bacterial cells and binds to RNA. Incubation with the Reagent takes up to 10 minutes and it is not clear, whether remaining viable cells would be able to uptake any nutrients even in case fresh LB medium was provided.

3.5.2 Use of 25×MIC of ampicillin

Prolonged exposure of bacteria to high concentrations of antibiotics, is a method used to induce formation of persisters and VBNC cells (**Keren et al., 2004; Orman and Brynildsen, 2013a**). In the work described here, the concentration of 25×MIC of ampicillin (125 µg/ml) was used, since it has been adopted by **Bamford et al., (2017)** for investigation of the VBNC phenotype in *E. coli* BW25113 grown the same stocks of as the stocks used for this work. This concentration is similar to the one reported by **Keren et al., (2004b)**: they used 100 µg/ml of ampicillin to induce persisters formation of *E. coli* HM21. In their experiments on persisters and VBNCs of *E. coli* MG1655, **Orman and Brynildsen (2013a)** used an even higher concentration of ampicillin: 200 µg/ml, corresponding to approximately 100×MIC value reported in the same paper.

Chapter 4: Conclusions and future developments

4.1 Profiling of gene expression of *E. coli* in lag phase

The first objective of this research project (Section 1.8) was investigating gene expression in a clonal population of wild type *E. coli* BW25113 (wt) during the lag phase of growth. RNA data for samples extracted from untreated cells at time points 0 and 1 hour, became part of the manuscript by **Smith et al., (2018)**. In order to gain a better understanding of the early lag phase, the data would require a deeper analysis than what has been done for the purpose of this dissertation. It would be necessary to take a systems biology approach and compare these data to databases mentioned in the paragraph 1.2, like UniProt (**The UniProt Consortium, 2019**), since bacterial genes are grouped in operons and constitute building blocks of signalling pathways. Here instead, a simplified approach was adopted: identifying the 15 most up- and down-regulated genes, compared to the time point 17 hours (stationary phase). The biological functions of the selected genes were then checked in the online database UniProt. This has shown that, during the lag phase, *E. coli* grown in low-salt LB adapts to the decrease of medium temperature, and to changes in medium pH, down-regulates sulphur metabolism, and genes associated with the stress response system RpoS.

Noteworthy, as shown in Figures 19 C and 20 C, there were 4 and 9 RNA extracts with a high RIN score for time point 0 and 1 hour respectively. In order to contain costs, it was decided to perform RNA-sequencing only on 3 extracts from each bacterial sample in order to have experimental triplicate of the bacterial transcriptome at each time point.

Potential future developments of investigating gene expression during the lag phase of wild type *E. coli* could include:

- Pooling more than one extract together in order to make the most of all the extracted samples. However, doing so might have introduced a further level of biological variation in the transcriptomic reads due to possible inter-culture variations (e.g. inoculum size, incubator temperature, media composition).

- Sequencing of RNA extracted from bacteria in exponential phase, grown in LB medium with different sodium chloride concentration, since the salt contents of rich media (Section 1.2) could have an impact on bacterial gene expression, for example of ompF (**Nikaido, 2003**) as mentioned in Section 3.3.2.
- Using LB medium which has been pre-warmed to the same temperature as the overnight culture. This may potentially change the expression pattern of the genes associated with cold shock (Section 3.3.1).
- Using media with well-defined composition such as M9 or MOPS to compare the transcriptomes from samples extracted at the same stages of growth in different media.

4.2 Investigating of gene expression of persisters and VBNC cells

The second objective of this research was to investigate gene expression in *E. coli* BW25113 (wt) persisters and VBNC cells. The protocol adopted here (Section 2.7) was based on methods described by **Keren et al., (2004b)**. The extractions proved to be challenging, the final RNA quantity varied each time, despite the same protocol was used. Attempts to extract RNA from 50 ml samples of the culture proved difficult due to cellular debris blocking the Qiagen spin column. Future work should begin from revision and improvements of RNA extraction protocol described in Section 2.7. Differences between the protocols compared in Table 6, like the choice of bacterial strain, method for isolating of the unlysed cells, ampicillin concentration or the timing of antibiotic addition, would all have to be considered in order to obtain more consistent results than those presented in Section 3.2.1.

4.2.1. Counting of persisters and VBNC

Liquid cultures of bacteria are not homogeneous (**Kragh et al., 2018**). Persisters and VBNC cells form stochastically in bulk cultures (**Lewis, 2007**) and are present before antibiotic pressure is applied (**Bamford et al., 2017**). Determining their proportions within the same culture, alongside with susceptible cells, would be an important element of future research. Quantification of numbers of persisters and of VBNC cells within the same bulk planktonic cultures, would require a combination of a few different experimental methods:

1. Flow cytometry on untreated bacterial samples fluorescently stained with green Syto9: quantification of the total number of bacterial cells per millilitre in exponential phase. Syto9 binds nucleic acids and penetrates both live and dead cells (**Bamford et al., 2017; Zhao et al., 2017**).
2. Flow cytometry on samples in exponential phase, treated with ampicillin for 3 hours, fluorescently stained with red Propidium Iodide (PI), which can only penetrate lysed cells (**Bamford et al., 2017; Zhao et al., 2017**).

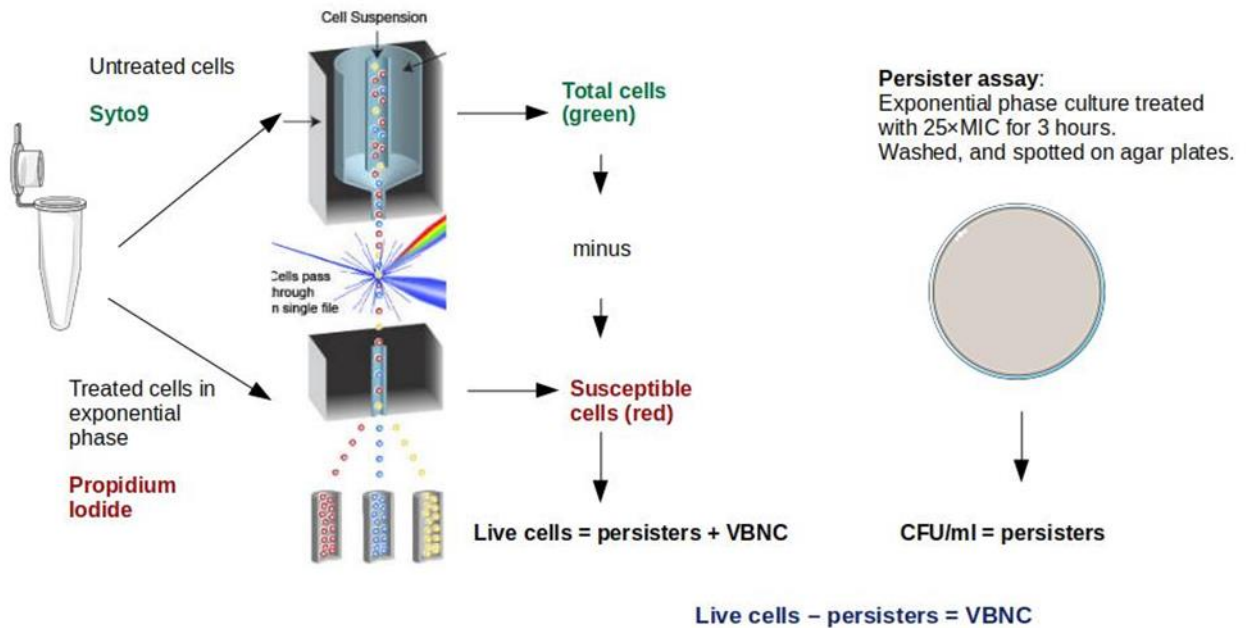
Appropriate gating of the fluorescently stained cells would allow to identify the numbers for the tolerant sub-populations (**Orman and Brynildsen, 2013a**), using the following relationship:

Green cells (total) – Red cells (susceptible) = Live cells (persisters + VBNC)

3. Bulk persisters assays, as described by **Keren and colleagues (2004a)** and summarised in [Figure 13](#): aliquots of bacterial culture in exponential phase, treated with high concentration of ampicillin (25×MIC) for 3 hours, washed and spotted on agar plates. The counts of CFU/ml give the fraction of persister cells.

Live cells – persisters = VBNC

Figure 29: Schematic workflow for experiments aimed to count persisters and VBNC cells in *E. coli* liquid cultures. Cartoons downloaded from: Overview of Flow Cytometry, Cell Signalling Technology, (https://www.cellsignal.co.uk/contents/_/overview-of-flow-cytometry/flow-cytometry-overview) and from Servier Medical Art (<https://smart.servier.com/>).



Precise timing of these experiments would have to be taken in account, since *E. coli* have a short doubling time (Orr, 2005). For example, the experiments could be performed by two researchers:

Researcher 1: Flow cytometry counts of total cells in exponential phase.

Researcher 2: Treatment of the culture with ampicillin.

After 3 hours of treatment:

Researcher 1: Flow cytometry counts of lysed cells.

Researcher 2: Persister assay.

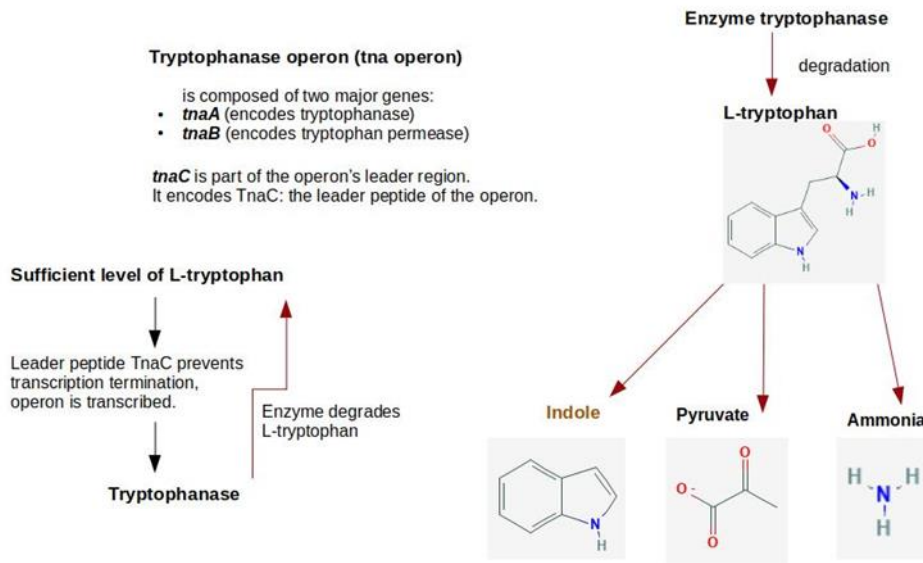
4.2.2. Investigating genes of interest

Some proteins have been associated with the phenomenon of persisters and VBNC in *E. coli*, as reviewed by **Maissoneuve and Gerdes (2014)** or **Lewis (2007)**. For example, up-regulation of TolC (Section 1.5.2; **Pu et al., 2016**), or of the Maz E - MazF toxin-antitoxin system (Section 1.7.1, **Cho et al., 2017**). Therefore, cross-referencing existing literature with RNA data for the tolerant sub-populations, could be the first step in further understanding of their gene expression at a given stage of growth. Influence of genes of interest on frequencies of persisters and VBNC cells, could be investigated by using knock-out mutant strains from the Keio collection. Their frequency of persisters and VBNC cells could be compared to the general population using the combination of flow cytometry and persister assays, as described above (Section 4.1.1).

4.2.3 Using fluorescent biomarkers

In their microfluidics Mother Machine experiments, **Bamford et al., (2017)** used promoters of some genes of interest, inserted into low copy number plasmids upstream of the GFP encoding gene (**Zaslaver et al., 2006**). This allowed them to monitor the promoter activity by measuring GFP fluorescence. **Bamford and colleagues (2017)** identified the *tnaC* reporter strain as a potential fluorescent biomarker of VBNC and persisters, which had decreased fluorescence compared to the rest of the population. The gene *tnaC* is part of the operon encoding tryptophanase, which in turn is necessary to produce the signalling molecule indole ([Figure 30](#)) associated with increased numbers of persisters in bulk cultures (Section 1.7).

Figure 30: Schematic of interactions between the different components of the *tnaC* operon in *E. coli*.

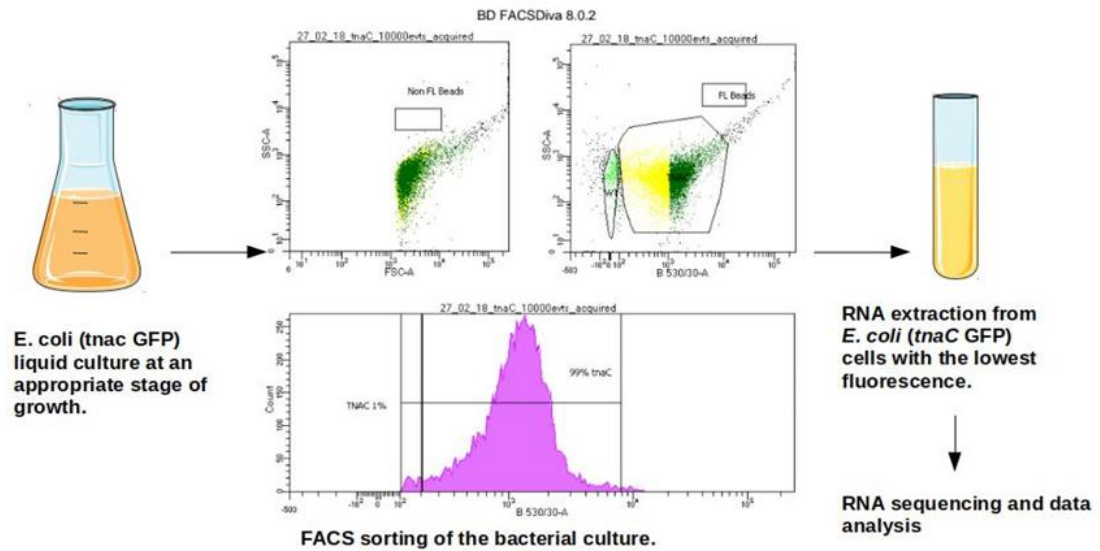


Such a fluorescent marker could potentially allow to isolate the tolerant phenotypes from the bulk of the population using FACS, with subsequent RNA extraction (Figure 31). The fluorescent strains would have to be first tested for proportions of persisters and VBNC cells in the exponential and in the stationary phases of growth. The growth stage of choice would have to be high in the numbers of tolerant cells, since:

- A.** their small numbers could cause sample loss during sorting,
- B.** it could considerably extend sorting times with potential changes in transcription patterns.

Another potential challenge would be the correct gating of the entire fluorescent population, in order to distinguish the cells of interest from the background noise, and a very limited sample volume for RNA extraction.

Figure 31: Schematic workflow of potential future experiments involving *E. coli* *tnaC* GFP reporter strain, grown in liquid culture and sorted using FACS. Cartoons downloaded from Servier Medical Art (<https://smart.servier.com/>). FACS graphs come from candidate's own work.



Transcriptomics data from bacterial RNA, extracted at a given stage of growth in liquid culture, allow to investigate gene expression in response to the changing environment. Persisters and VBNC cells are an inherent fraction of bacterial planktonic cultures (**Bamford *et al.*, 2017**). They can currently be isolated from the rest of the population only after an antibiotic treatment (**Keren *et al.*, 2004b**). Experimental challenges, for example a small sample quantity, or difficulties in physical separation of the lysed and unlysed cells after antibiotic challenge, did not allow to fulfil the aim of this dissertation (Section 1.8). However, the work described here, is a first step towards further studies of the tolerant sub-populations of laboratory-adapted wild type *E. coli*, grown in a common laboratory medium.

Supplementary Materials:

Table 1S. RNA samples for time points 0 and 1 hour, extracted from untreated *E. coli* aliquots of 0.5 ml, using spin columns Qiagen RNeasy Micro kits and cleaned with Agencourt RNA Clean XP beads. The samples were assessed for quantity and quality, as described in Section 2.2.

Sample ID	Sample concentration (ng/μl): Nano Drop	Nano Drop 260/280 ratio	Nano Drop 230/280 ratio	Sample concentration (ng/μl): Qubit	Agilent Tape Station RIN
Time 0 hours					
14/03/2017	1.6	1.41	1.49		4.5
11/04/2017	2.8	1.40	0.97		6.4
13/04/2017	2.6	1.55	1.17		6.8
04/05/2017 (1)	3.7	1.66	0.90		4.2
04/05/2017 (2)	3.8	1.73	0.33		4.5
08/05/2017 (1)	5.8	1.64	0.71		7.7
08/05/2017 (2)	3.3	1.83	0.77	0.58	7.7
09/05/2017 (1)	3.9	1.63	0.73	0.83	7.7
09/05/2017 (2)	4.3	1.37	0.60	0.61	8.0

Sample ID	Sample concentration (ng/μl): Nano Drop	Nano Drop 260/280 ratio	Nano Drop 230/280 ratio	Sample concentration (ng/μl): Qubit	Agilent Tape Station RIN
16/05/2017 (1)	0.9	0.50	0.79		5.1
16/05/2017 (2)	1.0	0.57	0.60		4.6
25/05/2017 (1)					5.1
25/05/2017 (2)					4.8
Time 1 hour					
14/03/2017	10.7	2.01	1.40	7.24	7.2
23/03/2017	7.2	2.07	1.44		8.2
30/03/2017	6.2	2.38	2.34		8.3
11/04/2017	6.3	1.82	1.57		7.9
12/04/2017	7.1	1.62	1.43	3.99	8.0
13/04/2017	3.2	1.68	1.27		
18/05/2017				2.0	
19/05/2017 (2)				3.58	7.8

Sample ID	Sample concentration (ng/μl): Nano Drop	Nano Drop 260/280 ratio	Nano Drop 230/280 ratio	Sample concentration (ng/μl): Qubit	Agilent Tape Station RIN
19/05/2017 (3)				4.24	8.2
23/05/2017 (1)				6.88	8.2
23/05/2017 (2)				6.19	8.1
24/05/2017 (1)				4.48	8.0
24/05/2017 (2)				5.99	8.1
08/08/2017 (1)	3.2	1.79	0.36	2.72	7.0
09/08/2017 (1)	14.3	1.56	0.52	7.05	8.1

Table 2S. List of *E. coli* RNA samples at time 1 hour, extracted using TRIzol™ Reagent from aliquots of 1 ml and 50 ml of liquid bacterial culture, as described in Section 2.3.

Sample ID	Sample concentration (ng/μl): Nano Drop	Nano Drop 260/280 ratio	Nano Drop 230/280 ratio	Sample concentration (ng/μl): Qubit	Agilent Tape Station RIN
Time 1 hour, aliquots 1 ml of culture					
27/07/2017	43.2	1.55	0.14	3.82	4.0
02/08/2017	8.9	1.52	0.07	11.3	4.6
04/08/2017	1.9	1.67	0.01	Out of range	2.5
Time 1 hour, aliquots 50 ml of culture					
27/07/2017	86.0	1.76	0.44	91	3.3
02/08/2017	35.5	1.59	0.25	53	1.1
04/08/2017	56.5	1.86	0.47	69	1.4

Table 3S. List of RNA samples for time point 4 hours, after 3-hour ampicillin challenge (25xMIC). The samples were extracted using Qiagen spin column kits, as described in section 2.7 of Chapter 2.

Sample ID	Sample concentration (ng/μl): Nano Drop	Nano Drop 260/280 ratio	Nano Drop 230/280 ratio	Sample concentration (ng/μl): Qubit	Agilent Tape Station RIN
Samples extracted from aliquots 0.5 ml of culture					
14/08/2017	69.9	2.06	1.03	58	7.9
16/08/2017	106.8	2.11	0.32	90.8	8.2
22/08/2017 (1)	60.1	1.96	0.72	47.4	6.8
22/08/2017 (2)	53.1	2.12	1.91	55.5	6.0
24/08/2017 (1)	59.9	1.97	0.58	70.8	7.3
24/08/2017 (2)	95.5	2.08	0.89	64.0	7.7
12/10/2017 (1)	107.1	2.12	0.24	97.2	
12/10/2017 (2)	216.0	2.09	0.71	240	
24/10/2017	696.2	2.10	1.13	700	

Sample ID	Sample concentration (ng/μl): Nano Drop	Nano Drop 260/280 ratio	Nano Drop 230/280 ratio	Sample concentration (ng/μl): Qubit	Agilent Tape Station RIN
27/10/2017	1068.0	2.10	2.17	936	
Samples extracted from aliquots 50 ml of culture					
14/08/2017	639.9	2.10	1.86	570	7.3
16/08/2017	439.7	2.06	2.13	360	7.7

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