PHENOTYPIC AND GENE EXPRESSION RESPONSES OF *E. COLI* TO ANTIBIOTICS DURING SPACEFLIGHT

by

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Phenotypic and Gene Expression Responses of *E. coli* to Antibiotics during Spaceflight

Thesis directed by Associate Professor Dr. David Klaus and Research Professor Dr. Louis Stodieck

Bacterial susceptibility to antibiotics has been shown *in vitro* to be reduced during spaceflight; however, the underlying mechanisms responsible for this outcome are not fully understood. In particular, it is not yet clear whether this observed response is due to increased drug resistance (a microbial defense response) or decreased drug efficacy (a microgravity biophysical mass transport effect). To gain insight into the differentiation between these two potential causes, an investigation was undertaken onboard the International Space Station (ISS) in 2014 termed Antibiotic Effectiveness in Space-1 (AES-1). For this purpose, *E. coli* was challenged with two antibiotics, Gentamicin Sulfate and Colistin Sulfate, at concentrations higher than those needed to inhibit growth on Earth. Phenotypic parameters (cell size, cell envelope thickness, population density and lag phase duration) and gene expression were compared between the spaceflight samples and ground controls cultured in varying levels of drug concentration. It was observed that flight samples proliferated in antibiotic concentrations that were inhibitory on Earth, growing on average to a 13-fold greater concentration than matched 1g controls. Furthermore, at the highest drug concentrations in space, *E. coli* cells were observed to aggregate into visible clusters. In spaceflight, cell size was significantly reduced, translating to a decrease in cell surface area to about one half of the ground controls. Smaller cell surface area can in turn proportionally reduce the rate of antibiotic molecules reaching the cell. Additionally, it was observed that genes – in some cases more than 2000 – were overexpressed in space with respect to ground controls. Up-regulated genes include *poxB*, which helps catabolize glucose into organic acids that alter acidity around and inside the cell, and the *gadABC* family

genes, which confer resistance to extreme acid conditions. The next step is to characterize the mechanisms behind the observed gene expression, its implications, and most importantly, how this knowledge can help prevent the acquisition and spread of antibiotic resistance in pathogens on Earth.

DEDICATION

I would like to dedicate this thesis to my parents, who gave the little they had to ensure I would have the opportunity of an education. Their efforts and struggles have allowed me to have a key to unlock the mysteries of our world, and beyond.

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ACRONYMS

API	Active Pharmaceutical Ingredient
ASTP	Apollo-Soyuz Test Project
BSL	BioSafety Level
CAL	Central Analytical Lab
CDC	Center for Disease Control and Prevention
CI	Confidence Interval
CRS	Commercial Resupply Services
FPA	Fluid Processing Apparatus
FSB	Fundamental Space Biology
HARV	High Aspect Rotating Vessel
HRR	Human Research Roadmap
ICE	Isolated Confined Environment
ISS	International Space Station
JSC	Johnson Space Center
LCMS	Liquid Chromatography Mass Spectrometry
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NASA	National Aeronautics and Space Administration
NIH	National Institutes of Health
OD	Optical Density
PD	Pharmacodynamics
RWV	Rotating Wall Vessel

SE	Standard Error
SMG	Simulated Microgravity
UPLC	Ultra-High Performance Liquid Chromatography
USP	United State Pharmacopeia

CHAPTER I

INTRODUCTION

Long-term exploration of space introduces numerous risks to astronauts' health with altered human-microbe interaction presenting a variety of concerns. The spacecraft itself creates an environmental niche for bacterial growth as well as for facilitating microflora exchange among its crew (Ilyin, 2005; Novikova, 2004; Taylor & Sommer, 2005). Additionally, astronauts' immune systems are thought to become suppressed, possibly due to factors such as stress, microgravity, or radiation (Borchers, Keen, & Gershwin, 2002; Stowe, Pierson, & Barrett, 2001). Bacteria also behave differently in space, most notably in terms of generally enhanced proliferation (Benoit & Klaus, 2007; Kacena, Merrell, et al., 1999; David Klaus, Simske, Todd, & Stodieck, 1997; Klaus, Luttges, & Stodieck, 1994; Tixador, Richoilley, Gasset, Planel, et al., 1985; Todd et al., 1998) and modified biofilm formation (Kim et al., 2013; McLean, Cassanto, Barnes, & Koo, 2001). Increased virulence, or capacity to cause disease, has been observed (Crabbe et al., 2011; Nickerson et al., 2000; Wilson et al., 2007), a thicker cell envelope was noted in one investigation (Tixador, Richoilley, Gasset, Planel, et al., 1985), and genetic recombination via conjugation has been shown to increase (Ciferri, Tiboni, Di Pasquale, Orlandoni, & Marchesi, 1986). Reduced bacterial susceptibility to antibiotics has also been documented for over three decades (Juergensmeyer, Juergensmeyer, & Guikema, 1999; Kacena & Todd, 1999; Kitts et al.,

2009; Klaus & Howard, 2006; Lapchine et al., 1986, 1987; Moatti et al., 1986; Parra, Ricco, Yost, McGinnis, & Hines, 2008; Ricco et al., 2010; Tixador et al., 1994; Tixador, Richoilley, Gasset, Planel, et al., 1985; Tixador, Richoilley, Gasset, Templier, et al., 1985). Finally, medication shelf life has been shown to be reduced in space by up to 1/3 the usable life on Earth (Du et al., 2011) and there is evidence that the bioavailability of orally-administered drugs may be decreased in space (Tietze & Putcha, 1994).

Besides the problems that each of these responses might present individually, they potentially could interact to negatively impact the health of astronauts. For example, an increase in genetic recombination raises the probability of antibiotic resistant traits being transferred from one bacterial strain to another. Increased microflora exchange facilitates the transmission of these antibiotic-resistant strains from one crewmember to another, as has been documented to occur in Soviet space stations and at the International Space Station (ISS) (Ilyin, 2005). Increased bacterial virulence and decreased astronaut immune function produce conditions more likely to cause bacterial infection. A correlation between antibiotic resistance and increased cell wall thickness – both documented in separate spaceflight studies – has been observed terrestrially (Sieradzki & Tomasz, 2003). Furthermore, reduced susceptibility to antibiotics and reduced medication shelf life could exacerbate treatment of infections.

From this comes the problem statement for this dissertation: "*Astronaut's health is jeopardized by observed increase in bacterial proliferation and reduced susceptibility to antibiotics*". The significance of this problem is not only stressed throughout the literature referred to in this thesis but has also been identified by the National Aeronautics and Space Administration (NASA) in the Human Research Roadmap (HRR), the Fundamental Space Biology (FSB) Plan and other NASA documents and reports (Galvez, 2013; Tomko, Sun, & Quincy, 2010; Watkins, 2010).

Terrestrially, drug resistance is a problem that has been rapidly increasing worldwide during the last couple of decades. For example, in 1992 13,300 patients died from multi-drug resistant bacteria acquired in hospitals; that number increased to 90,000 by 2012 (NIH, 2012). The Center for Disease Control and Prevention (CDC) reports that this problem is costing the U.S. government $20 billion in excess health care cost and $35 billion in societal costs (CDC, 2011).

Understanding the exacerbated bacterial response of decreased susceptibility to antibiotics in spaceflight can be used to gain insight aimed at reducing drug resistance acquisition on Earth (Klaus & Howard, 2006). However, the underlying mechanisms responsible for enabling bacterial proliferation in normally inhibitory levels of antibiotics observed to occur in space are not fully understood. In particular, it is not yet clear whether this response is due to increased drug resistance (a microbial defense response) or decreased drug efficacy (a microgravity mass transport biophysical effect). One of the goals of this doctoral work is to differentiate between these two phenomena, which could help gain insight into the causal mechanisms behind decreased bacterial susceptibility to antibiotics here on Earth. The core of this dissertation is an investigation undertaken onboard the International Space Station, termed Antibiotic Effectiveness in Space-1 (AES-1), which was launched on Orbital Commercial Resupply Services CRS-1 in January, 2014. The hypothesis behind this experiment was that antibiotics used to inhibit bacteria grown in space would exhibit reduced efficacy compared to 1g controls and would be associated with specific changes in bacterial gene expression that correlate with phenotypic changes and cell survival. To test this hypothesis, *E. coli* was challenged with two antibiotics, Gentamicin Sulfate and Colistin Sulfate, at concentrations higher than those needed to inhibit growth on Earth. The samples were fixed at completion of the experiments to avoid bacterial re-adaption to gravity. At their return to Earth, spaceflight (and their matched

ground control) samples were later assessed for changes on several phenotypic parameters (cell size, cell envelope thickness, population density and lag phase duration) and gene expression, and related to bacterial populations achieved in varying levels of inhibitory drug concentrations.

1.1 PROBLEM STATEMENT

This thesis focused on the following problem:

Astronaut's health is jeopardized by observed increase in bacterial proliferation and reduced susceptibility to antibiotics.

For this purpose, an investigation was undertaken onboard the International Space Station (ISS): AES-1. In it, *E. coli* was challenged with two antibiotics, Gentamicin Sulfate and Colistin Sulfate, at concentrations higher than those capable of inhibiting growth on Earth. Phenotypic parameters (cell size, cell envelope thickness, population density and lag phase duration) and gene expression were compared between the spaceflight samples and ground controls cultured in varying levels of drug concentration.

1.2 RATIONALE

The importance of this study comes mainly from the implications the above-mentioned phenomena could have for astronauts on long-term space missions. Additionally, there is another phenomenon occurring on Earth that is related: the rise of new, drug resistant strains of bacterial pathogens. This thesis work may also allow us to gain insights that may be applicable to clinical antibiotic research for Earth applications.

1.2.1 Implications in Space

All of the observed spaceflight changes in bacterial behavior, such as increased virulence, proliferation, mutation rate, and cell envelope thickness raise potential concern for human space programs. These problems are further exacerbated by other observed phenomena such as astronaut immunosuppression, increased microflora exchange, and reduced antibiotic shelf life and efficacy. All of these aspects together may create the perfect storm for potential bacterial infection during spaceflight, which may be complicated to treat.

NASA's Human Research Roadmap (HRR) lists a series of risks to future human exploration of space. Two of these risks are related with this thesis work, namely 1) risk of clinically relevant unpredicted effects of medication, and 2) risk of adverse health effects due to alteration in host-microorganism interactions. The first is described as being based on our lack of knowledge on a) pharmacodynamics and on b) drugs' effectiveness on microbes altered by spaceflight (Gaps Pharm04 and Pharm05, respectively) (Galvez, 2013). The second risk comes from the observed alterations in microbial virulence and astronaut immunosuppression. It drives the "host-microbe virulence – cellular studies" task, which aims at determining the microbial responses to spaceflight that may have an impact on infectious diseases during spaceflight missions.

NASA's Fundamental Space Biology (FSB) Plan 2010-2020 identifies the following as one of the overarching questions that will guide FSB science direction "Are decreased mass transfer, or physical force changes in membranes and cell walls, the main effect of microgravity on the cell?" (Tomko et al., 2010). Furthermore, NASA's Space Medicine Exploration report stresses the importance of being able to address in-flight infectious conditions (Watkins, 2010); similarly, (Taylor & Sommer, 2005) explain the importance of knowing how much antibiotic is needed to inhibit bacterial growth in space, as this will be

needed to address lacerations and open fractures which are likely to occur during surface extra-vehicular activities (Zea, Diaz, Shepherd, & Kumar, 2010). The study of infectious diseases in space, and of microbial responses to spaceflight and their implications on the host may not only mitigate risks for future human space exploration but may also "open a new chapter in the understanding of health and disease to benefit the general public" (Phys, 2013).

1.2.2 Implications on Earth

Acquiring new knowledge on increased bacterial proliferation and decreased susceptibility to antibiotics in space may allow us to gain insight on the causal mechanisms behind these phenomena (Klaus & Howard, 2006). These authors indicate that, if antimicrobial resistance is in fact enhanced in spaceflight as suggested in the literature, this might help us better understand the underlying causal mechanisms of the resistance acquisition process. Furthermore, they indicate that the overall investigations of microbial responses to spaceflight may provide us with knowledge applicable to related clinical research on Earth. Ideally, the results from this work can provide new insights for potential drug development.

Although the production of new drugs is important, during the last decades there has been a steady decline in new antibacterial drugs entering the market around the world (Chopra, Hodgson, Metcalf, & Poste, 1997; ECDC, 2009; Spellberg et al., 2008; Tatfar, 2011). This is in part due to the lower financial returns pharmaceutical companies receive for these drugs compared to the ones that treat chronic diseases. On the other hand, Tatfar (2011) states that, in order to address the antimicrobial resistance problem, not only new drugs need to be developed but that we also need to understand how bacteria develop resistance mechanisms against drugs.

Microbes do indeed find ways around drugs. Mutation and the acquisition of resistance genes from other organisms have allowed bacteria to develop resistance to existing antibiotics (Reed, Barrett, Threlfall, & Cheasty, 1995). Improper use of antibiotics, e.g. when patients don't take the full doses they were prescribed, is exerting a selective pressure on bacteria that favors the growth of the drug-resistant strains (Tatfar, 2011).

These drug-resistant strains then find their ways from one place to the other (Johnson et al., 1999; Zell & Goldman, 2007). A group of British geneticists found that a strain of *C. difficile* became resistant to antibiotics in two different ways: by acquiring a single mutation in an enzyme that binds fluoroquinolones (a class of antibiotics) and by capturing genes that pump antibiotics out of their cells. *Clostridium difficile* infections are common in hospitals where patients have been treated with large doses of antibiotics (He et al., 2013). This British group also discovered that in 2002, an antibiotic resistant strain of *C. difficile* arose in the U.S. and a couple of years later, it was present in Europe, Australia and Asia. This is a problem to many people as antibiotic resistant *Clostridium difficile* kills nearly 14,000 people a year in the U.S. alone (He et al., 2013).

During the last decade an increase on drug-resistant strains of bacteria has shown up in hospitals around the world. In the United States, between 5 and 10 percent of patients develop a bacterial infection during their stay in the hospital produced by new drug-resistant strains. The United States government spends over $20 billion a year in excess health care costs stemming from drug resistant bacteria (CDC, 2011). Beyond and above the financial cost is the invaluable human cost. In the U.S. alone, about 90,000 people die every year from drug resistant bacteria (NIH, 2012), (about one fifth of deaths are due to the 3rd generation cephalosporin-resistant *E. coli* (WHO, 2012)). This is a steep increase from 13,300 patient deaths in 1992 (NIH, 2012).

1.3 THESIS OBJECTIVES

This main goal of this thesis is

To characterize phenotypic and gene expression changes in E. coli that permit proliferation in space in antibiotic concentrations that one Earth would be inhibitory.

This is with the ultimate goal of helping in the fight against drug-resistant bacteria on Earth. This was done through four major aims:

A1. Identify if there are changes in *E. coli*'s a) cell size, b) cell envelope thickness, and c) final cell counts when challenged with antibiotic in space, compared to 1g controls.

A2. Verify that *E. coli* will proliferate under normally (1g) inhibitory concentrations of two different antibiotics in microgravity.

A3. Identify if there is a correlation between *E. coli* cell size, population growth dynamics, cell envelope thickness and bacterial susceptibility to various antibiotics in microgravity.

A4. Assess if there are any correlations between *E. coli* gene expression and bacterial susceptibility to the specified antibiotics in microgravity.

1.4 HYPOTHESES

The overarching hypothesis is

Antibiotics used to treat bacteria grown in space will exhibit reduced efficacy and will be associated with specific changes in bacterial gene expression that correlate with cell survival.

Specifically, this hypothesis is broken down in a set for Gentamicin (labeled with "G") and a set for Colistin Sulfate (labeled with "C")"

1.4.1 Antibiotic: Gentamicin Sulfate

1.4.1.1 Morphology and Physiology Hypotheses

G1: When challenged with Gentamicin Sulfate in microgravity, *E. coli* ATCC 4157 cells will grow to larger sizes compared to matched 1g controls.

G2: When challenged with Gentamicin Sulfate in microgravity, *E. coli* ATCC 4157 cells will develop thicker cell envelopes compared to matched 1g controls.

G3: When challenged with Gentamicin Sulfate in microgravity, *E. coli* ATCC 4157 cells will grow to higher final cell concentrations compared to matched 1g controls.

G4: When challenged with Gentamicin Sulfate in microgravity, *E. coli* cells ATCC 4157 will have reduced lag phases compared to matched 1g controls.

1.4.1.2 Antibiotic Effectiveness Hypotheses

G5: In microgravity, *E. coli* ATCC 4157 will proliferate under normal (1g) inhibitory concentrations of Gentamicin Sulfate.

1.4.1.3 Relation between interrogated parameters

G6: There is a correlation between population growth dynamics, cell size, and cell envelope thickness of *E. coli* ATCC 4157, and bacterial susceptibility to Gentamicin Sulfate.

1.4.2 Antibiotic: Colistin Sulfate

1.4.1.1 Morphology and Physiology Hypotheses

C1: When challenged with Colistin Sulfate in microgravity, *E. coli* ATCC 4157 cells will grow to larger sizes compared to matched 1g controls.

C2: When challenged with Colistin Sulfate in microgravity, *E. coli* ATCC 4157 cells will develop thicker cell envelopes compared to matched 1g controls.

C3: When challenged with Colistin Sulfate in microgravity, *E. coli* ATCC 4157 cells will grow to higher final cell concentrations compared to matched 1g controls.

C4: When challenged with Colistin Sulfate in microgravity, *E. coli* cells ATCC 4157 will have reduced lag phases compared to matched 1g controls.

1.4.1.2 Antibiotic Effectiveness Hypotheses

C5: In microgravity, *E. coli* ATCC 4157 will proliferate under normal (1g) inhibitory concentrations of Colistin Sulfate.

1.4.1.3 Relation between interrogated parameters

C6: There is a correlation between population growth dynamics, cell size, and cell envelope thickness of *E. coli* ATCC 4157, and bacterial susceptibility to Colistin Sulfate.

CHAPTER 2

BACKGROUND

When Jean-Loup Chrétien launched to the USSR's Salyut 7 Space Station, he was carrying some of his own microflora in glass ampoules. Soviet scientists collected and isolated *E. coli* and *Staphylococcus aureus* bacteria from Chrétien before launch. Spationaut Chrétien, the first Frenchman to go to space, made observations on the bacteria that used to live in him to search for changes due to spaceflight. These observations were part of the CYTOS 2 experiment and led to two remarkable findings. After growing the bacteria in space, the French team of scientists behind Chrétien's experiment observed that the inflight *E. coli* needed more than four times as much antibiotic for growth to be inhibited with respect to ground controls. Additionally, the French scientists from the Université Paul Sabatier discovered that *S. aureus* spaceflight cultures had greater cellular envelope thickness. Post-flight analysis showed no modification on the antibiotic sensitivity or biochemical characters relative to ground controls, indicating that the changes seen in space were not acquired characters. The French team finally hypothesized that the increased antibiotic resistance observed in space may be explained by a stimulating effect of cellular multiplication and by the greater thickness of cellular envelope structure, which

reduces antibiotic penetration (Tixador, Richoilley, Gasset, Planel, et al., 1985). This hypothesis is congruous with Fick's law of diffusion.

This Franco-Soviet experiment was not unique in its results. American scientists also flew bacteria near the beginning of the space program. American space-bound bacterial experiments include Biosatellite II (1967), Apollo 16 (1972), Skylab 2 (1973), Skylab 4 (1973-4), and Apollo-Soyuz Test Project (ASTP) (1975). From these, Biosatellite II and Skylab 2 carried *E. coli*. (Klein, 1981)

Biosatellites I and II carried two bacterial species to space: *Salmonella typhimurium* and *E. coli*. In 1966, Biosatellite I failed to re-enter Earth as planned so the bacterial data was lost. In 1967, the Biosatellite II experiment led by R.H.T. Mattoni, assessed the impact of spaceflight and gamma irradiation on bacterial growth (Parfenov & Lukin, 1973; Souza, Hogan, & Ballard, 1995). Mattoni et al. (1968) concluded that spaceflight resulted in greater bacterial densities, most likely due to microgravity. They also concluded that this was because the random distribution of the cells in the liquid medium allowed them to access nutrients better, and transported waste away from the cells more efficiently (Souza et al., 1995).

An experiment flown to Skylab in 1977 was designed to, among other objectives, determine the effects of microgravity on *B. subtilis* and *E. coli*'s growth rate. The experiment failed because of the loss of Skylab's meteoroid shield, which caused the space station to overheat. This experiment was repeated on Skylab 3 but *E. coli* was replaced for *Bacillus mycoides*. That experiment provided data from which it was concluded that cultures grown in Skylab were usually larger and grew faster relative to their controls on Earth (Summerlin, 1977).

Bacteria collected *in vivo* during the Apollo-Soyuz mission also demonstrated a higher antibiotic resistance than the bacteria collected pre and post-flight (Taylor & Zaloguev, 1978). Similarly, in October 1985, Space Shuttle Challenger took to orbit the D-1 Spacelab with *E. coli* and the Colistin antibiotic, and similar results were obtained (Moatti et al., 1986). These and related studies provide the basis of this thesis's work.

Several other phenomena occur in space that give this research significance. Astronaut's health may be in jeopardy not only because of reduced antibiotic efficacy but due to myriad situations characteristic of spaceflight, which are categorized as seen in Table 1.

Table 1. Phenomena observed in spaceflight that may jeopardize astronauts' health.

Phenomenon observed in space	References
Increased bacterial virulence	(Cameron, Howden, & Peleg, 2011; Matin, Lynch, & Benoit, 2007; Mermel, 2013; Nickerson et al., 2000; Rosenzweig et al., 2010; Tomasz, 1994; Wilson et al., 2007)
Increased bacterial mutation rate	(Tatfar, 2011; Taylor & Sommer, 2005)
Increased bacterial proliferation	(Benoit & Klaus, 2007; Bhaskaran, Dudhale, Dixit, Sahasrabuddhe, & Vidyasagar, 2011; He et al., 2013; Kacena, Merrell, et al., 1999; Klaus et al., 1997; Klaus et al., 1994; Klaus & Howard, 2006; Mermel, 2013; Nickerson et al., 2000; Tixador, Richoilley, Gasset, Planel, et al., 1985; Todd et al., 1998)
Increased cellular envelope thickness	(Tixador, Richoilley, Gasset, Planel, et al., 1985)
Reduced bacterial susceptibility to antibiotics	(Benoit & Klaus, 2007; Ilyin, 2005; Kitts et al., 2009; Klaus & Howard, 2006; Mermel, 2013; Moatti et al., 1986; Parra et al., 2008; Ricco et al., 2010; Taylor & Sommer, 2005; Tixador, Richoilley, Gasset, Planel, et al., 1985)
Reduced antibiotic shelf life	(Du et al., 2011; Klaus & Howard, 2006; Moatti et al., 1986; Tietze & Putcha, 1994)
Increased microflora exchange	(Ilyin, 2005; Klaus & Howard, 2006; Mermel, 2013; Novikova, 2004; Taylor & Sommer, 2005)
Astronaut immunosuppression	(Borchers et al., 2002; Mermel, 2013; Stowe et al., 2001; Tietze & Putcha, 1994; Todd et al., 1998)
Improved biofilm formation	(Kim et al., 2013)

First, from the astronaut side, immunosuppression and increased microflora exchange can be identified. Second, from the antibiotics side, reduced shelf life, and reduced efficacy are noted. Third, from the bacterial side, several phenomena have been observed: increased virulence, increased genetic recombination via conjugation, increased proliferation and increased cell envelope thickness.

2.1 ASTRONAUT-RELATED PHENOMENA

2.1.1 Astronaut Immunosuppression

It is known that the space environment impacts the human immune system (Borchers et al., 2002; Mermel, 2013). It is not clear if this is a product of radiation, stress characteristic of spaceflight, living in an Isolated Confined Environment (ICE), or other physiological and/or psychological stressors. However, several of the effects the immune system goes through during spaceflight have been identified. Mermel (2013) summarized these effects as follows: “impaired wound healing, inhibition of leukocyte blastogenesis and altered leukocyte distribution, altered monocyte and granulocyte function, impaired leukocyte proliferation following activation, altered cytokine production patterns, abrogated bone marrow responsiveness to colony-stimulating factors, altered T-cell intracellular signaling, inhibition of natural killer cell activity, inhibition of delayed-type hypersensitivity, and apparent Th2 potential bias shift during prolonged space travel”. Another literature survey conducted by (Borchers et al., 2002), where 335 papers were analyzed, came to similar conclusions. Immunosuppression may also increase the risk of infection and of herpesviruses reactivation, and diminishes anaerobic colonic flora (Mermel, 2013). Finally, inhibition of T-cell blastogenesis has also been documented (Todd et al., 1998).

2.1.2 Increased Microflora Exchange

Increased microflora exchange during spaceflight occurs, in part, due to the confined environment in which astronauts find themselves. This problem is of importance due to the fact that human microflora include drug resistant bacteria.

For example, Professor V.K. Ilyin (2005) of the Russian Academy of Science reported that during a 96-day spaceflight, a cosmonaut was administered ampicillin and eventually ampicillin-resistant *Staphylococci* were detected on a second cosmonaut. He hypothesized that an ampicillin-resistant strain of *Staphylococcus* was developed in the first cosmonaut and later transmitted to the second space traveller. This case exemplifies what the Russian professor states are the main etiological agents of infectious disease in an enclosed environment: human microflora and cross infection. He adds that some of the most likely bacteria to trigger infectious diseases are *Staphylococci, Streptococci* and representatives of the *Enterobacteriaceae* family such as *E. coli*.

Professor Ilyin's finding about the drug resistant bacteria exchange among cosmonauts came as part of a larger investigation where he compared the microflora of the crews aboard the Salyut 6, 7, and Mir Space Stations. He saw a systematic shift in their microflora. Dysbacteriosis, or an imbalance in the person's microflora in which opportunistic pathogenic bacteria (e.g. *E. coli*) increase in numbers while non-pathogenic bacteria decreases, was observed during these three space missions. Ilyin also described a direct relationship between mission duration and dysbacteriosis severity: D1 (least severe form of) dysbacteriosis was common in cosmonauts in 30-day missions whereas D2-D4 (most severe forms of) dysbacteriosis were observed in 63- and 96-day missions. No individuals from these flights presented dysbacteriosis-free microflora (Ilyin, 2005).

A review conducted by Klaus & Howard (2006) found that multiple other studies concur with Ilyin's results. For example, Taylor & Sommer (2005) mention that a large pool

of microbial genotypes will fly to space with the crew. From this pool, it is likely that antibiotic-resistant strains will emerge. Similarly, Mermel (2013) summarized several investigations that conclude that transmission of microbial flora – including multi-drug resistant pathogens – has been demonstrated to take place in the International Space Station.

2.2 BACTERIA-RELATED PHENOMENA

Decades of microbiological research in space have produced myriad results in terms of changes observed in bacteria during spaceflight. To give this rather extensive list some order, in this thesis they have been categorized under four groups: growth, mutation rate, cell envelope thickness and virulence.

2.2.1 Growth

Bacterial growth is distinguished in six phases: lag, acceleration, exponential, retardation, stationary and phase of decline (Monod, 1949). However, this categorization is usually simplified to three phases: lag phase, exponential growth phase and stationary phase. The duration of the first stage is driven by nutrient composition and the age and size of the inoculum. The duration of the second phase is dictated by nutrient/toxic byproduct concentrations around the cells (Klaus & Howard, 2006).

E. coli ATCC 4157, the same strain used for this thesis, was grown on seven different Space Shuttle flights under similar conditions (Klaus et al., 1997). It was concluded from these studies that spaceflight affected bacterial growth and resulted in three main changes: 1) reduced lag phase, 2) increased exponential growth time (2-5 hours longer) and 3) increased final cell count (72% average increase, n = 40 flight, 25 ground, $P<0.05$) (Klaus et al., 1997). However, final population increases of up to 257% were

observed. Starker increases were observed on cultures where bacteria had to adapt to a new nutrient medium. On experiments more closely comparable to the ones conducted for this thesis, Klaus et al. (1994) found that cultures grown in Medium E with 5 g/L glucose, had final populations between 20% and 104% larger relative to their ground controls—with the exception of one culture that had an 18% decrease (n=28, 14 flight, 14 ground) (Klaus et al., 1994). In general, several investigations have observed shorter lag phases and/or higher final cell counts (Bhaskaran et al., 2011; Brown, Klaus, & Todd, 2002; Kacena, Merrell, et al., 1999; Nickerson et al., 2000; Todd et al., 1998).

In a separate publication, Klaus et al. reviewed related literature and found seven investigations that concurred that microorganisms proliferated in space compared to their ground controls. In the same paper, Klaus also found two studies that reported no change in bacterial growth (Klaus, Simske, Todd, & Stodieck, 1997).

Another literature review, this one conducted by Mermel (2013), summarized some of the observed effects of spaceflight on bacteria, including enhanced growth patterns in liquid media, shortened lag phase and enhanced exponential growth. Other investigations also report similar results (Benoit & Klaus, 2007; Tixador, Richoilley, Gasset, Planel, et al., 1985). Speaking more closely of gram-negative bacteria (such as *E. coli*), Mermel (2013) also mentions increased density of such bacteria on the skin, upper airway and colon.

2.2.2 Mutation Rate

As bacteria reproduce, mutations occur. Some of these mutants protect the bacterium against a specific antibiotic. Once most of the bacteria die due to the antibiotic, the mutant remains to reproduce and replicate the resistance; this is known as antimicrobial selection pressure (Tatfar, 2011). Selective antibiotic pressure has indeed allowed bacteria to evolve defense mechanisms such as bypassing a metabolic step, to

prevent the antibiotic to reach its target, or by the production of an enzyme to break down the antibiotic before it attacks the cell (Taylor & Sommer, 2005). This selective antibiotic pressure may occur either before or during spaceflight.

Bacterial mutation has been observed to increase during long-term spaceflight (compared to Space Shuttle sortie flights of ~two weeks). During a 40 day Mir mission, yeast was noted to mutate at a rate between two and three times higher than the ground samples. Additionally, an Italian team of researchers reported that, in comparison to ground controls and flown 1 g centrifuge controls, three to four times more *E. coli* recombinants were produced in microgravity (Ciferri et al., 1986)..

2.2.3 Cell Envelope Thickness

The French team that flew the experiment in Salyut 7 and found that space bound *E. coli* required more than four times as much antibiotic as necessary on Earth to achieve growth inhibition, and that *S. aureus* had increased cell envelope thickness in microgravity, made several recommendations for future work. One of their recommendations was that, in order to understand the mechanisms behind increased antibiotic resistance in microgravity, it is necessary to investigate if the changes on cellular envelope are located on the membrane, the cell wall or the whole envelope (Tixador, Richoilley, Gasset, Planel, et al., 1985). Lamentably, literature on this topic is not as abundant as other of the topics covered in this thesis. However, a few papers do examine cell envelope thickness (Mermel, 2013; Moatti et al., 1986). Actually, in 1985 (Moatti et al., 1986) flew *E. coli* on the Space Shuttle Challenger and divided the cultures in two groups: one was centrifuged to 1 g while the other was left under normal microgravity conditions. They saw that both of these sets required twice the concentration of antibiotics, but there were no differences between the two flown sets. They concluded that the decrease in susceptibility to antibiotics could have

been due to changes in cell envelope permeability. It should also be mentioned that, on a separate line of research, a correlation between antibiotic resistance and increased cell wall thickness has been observed here on Earth (Sieradzki & Tomasz, 2003). This topic is investigated in more detail in Chapter 4.

2.2.4 Virulence

Increased virulence has been observed in *Salmonella enterica serovar Typhimurium* grown in rotating wall vessels. This was proved by infecting mice with 1 g bacteria and with bacteria grown in simulated-microgravity (SMG). Ten days after infection, 80% of the mice infected with SMG bacteria died versus 40% of those infected with 1-g bacteria (Nickerson et al., 2000). Similar results were observed (70% vs. 20%) with bacteria actually flown in space (Wilson et al., 2007). Other studies have reported increased bacterial virulence during spaceflight (Mermel, 2013; Rosenzweig et al., 2010) while others also relate changes in virulence with a bacteria's antibiotic resistance acquisition (Cameron et al., 2011; Matin et al., 2007; Tomasz, 1994).

2.3 ANTIBIOTIC-RELATED PHENOMENA

2.3.1 Antibiotic Effectiveness

Two classical approaches for quantifying bacterial sensitivity to antibiotics are 1) to define the minimal inhibitory concentration (MIC) of antibiotic and 2) to grow bacterial cultures in a bacteriostatic concentration of antibiotic and count the number of surviving cells (Moatti et al., 1986).

Tixador and his team flew another experiment after their CYTOS 2 experiment on Salyut 7 (Moatti et al., 1986). This newer experiment was aboard Space Shuttle Challenger

in the STS-61-A mission. Tixador's team measured *E. coli*'s sensitivity to antibiotic (Colistin) by comparing the MIC at spaceflight versus that of ground controls. They observed that in space, at least twice the amount of antibiotic was needed to inhibit the bacterial growth. The actual antibiotic concentration needed to inhibit bacterial growth in space could not be found due to the unsuccessful inoculation of the cultures with higher amounts of antibiotic.

Several other experiments and reviews have also indicated that greater concentrations of antibiotics are needed to inhibit bacterial growth in space (Benoit & Klaus, 2007; Klaus & Howard, 2006; Mermel, 2013; Taylor & Sommer, 2005; Tixador, Richoilley, Gasset, Planel, et al., 1985). One study concluded that antibiotic resistance goes up in people living in confined environments within 7 to 10 days of isolation; interestingly, this is also the peak of cross contamination or microflora exchange among astronauts (Ilyin, 2005). Beyond bacteria, similar results have been observed with yeast. A team of scientists from NASA Ames reported that *S. cerevisiae* grown in 4xMIC in space still presented metabolic activity in space during the PharmaSat experiment (Kitts et al., 2009; Parra et al., 2008; Ricco et al., 2010).

2.3.2 Antibiotic Shelf life

A point that must be added to the antibiotic susceptibility discussion is that it has been observed that spaceflight reduces antibiotics' shelf life (Du et al., 2011; Tietze & Putcha, 1994). Shelf life is defined as the duration a drug will last with at least 90% of its labeled potency. After comparing 35 formulations aboard the ISS to ground controls, Du et al. (2011) found that a higher percentage of medications from the station's kits had lower active pharmaceutical ingredient (API) respective to ground controls. Du et al. (2011) also concluded that the observed API reduction in space was a function of time and independent

of drug expiration date or dosage form (solid, semisolid or liquid). For example, promethazine, a medication against motion sickness, showed to stay within United States Pharmacopeia (USP) acceptability range after 600 days on Earth but only 200 days in space. Similarly, Amoxicillin remained well within USP acceptable range after 900 days on Earth but lasted less than 600 days in space. It is hypothesized that reduced antibiotic shelf life in space occurs due to chronic low dose of ionizing radiation or repackaging of drugs in flight-specific dispensers (Du et al., 2011).

2.4 RISK OF INFECTIOUS DISEASE DURING SPACEFLIGHT

Closed environments, such as spacecraft have proven to be niches for microbial proliferation – on surfaces, air and condensate (Novikova, 2004). This increases the risk of infectious diseases. A literature survey written by Mermel et al. (2013) summarized flight information relating to infectious diseases during spaceflight based on 742 different crewmembers of 106 Space Shuttle flights. He found that in total, there were 29 reported infectious disease incidents. This means that about 4% of the astronauts who were part of this investigation suffered some sort of infectious disease incident. Separately, it has also been documented that out of 28 analyzed Shuttle astronauts with latent herpesviruses, 11 had them reactivated in space (Borchers et al., 2002). Some of these cases were in spaceflight as short as 9 days. These 11 astronauts showed a 220% and 100% increase in adrenaline and noradrenaline, respectively (Stowe et al., 2001). A different study reported that during the first 33 Space Shuttle flights, over 500 individual doses of 31 different medications were taken (Tietze & Putcha, 1994). This represents 88% of the astronauts of those flights, with the caveat that these medications include treatments against motion sickness, sleeplessness, and other non-infectious diseases. A reportedly serious bacterial (*P. aeruginosa*) infection occurred on the Apollo 13 mission (Aviles, Belay, Fountain, Vance,

& Sonnenfeld, 2003) and the in-flight antibiotic treatment given during the mission was ineffective (Benoit, 2005). All this data serve as a basis to understanding the probability of different diseases from occurring during space travel. However, it must be stressed that these statistics are based on spaceflights of about two weeks – the longest space shuttle flight, STS-80 had a duration of 17.6 days while the shortest, STS-93 was in space for less than 5 days (Petty, 2005). These problems could be exacerbated during long-term spaceflight missions. For example, a round trip mission to Mars would take well over a year and these missions would likely include surface Extra-Vehicular Activities (EVA). Surface EVAs increase the probability of lacerations and fractures that could results in serious bacterial infections (Zea, Diaz, Shepherd, & Kumar, 2010). For all these reasons, it is paramount that we learn how to mitigate bacterial infections and risk of bacterial transmission in space (Mermel, 2013). Beyond that, the risk of infection is aggravated by the risk of inefficient antibiotic treatments and the risk of the rise of drug-resistant bacteria.

2.5 CURRENT HYPOTHESES BEHIND THE PHENOMENA INVESTIGATED IN THIS THESIS

A thorough literature survey has allowed for a compilation of the most common acting mechanisms behind currently established hypotheses on why bacteria grow differently in space as well as the observed decrease in antibiotic effectiveness during spaceflight. These mechanisms are:

1. Extracellular environment and mass transport (Benoit, Brown, Todd, Nelson, & Klaus, 2008; Benoit & Klaus, 2007; Kitts et al., 2009; Klaus, 2004; Klaus, Benoit,

Nelson, & Hammond, 2004; Klaus et al., 1997; Klaus & Howard, 2006; Nicholson et al., 2011; Ricco et al., 2007, 2010; Todd & Klaus, 1996).

2. "R-plasmid transmission" (Boever et al., 2007; Ilyin, 2005; Mermel, 2013).
3. Changes to cellular envelope in space (Mermel, 2013; Moatti et al., 1986; Tixador, Richoilley, Gasset, Planel, et al., 1985).
4. Quorum sensing (Lee & Collins, 2011; Shapiro, 1998; Vega, Allison, Khalil, & Collins, 2012).

The first and last of these hypotheses try to explain both, bacterial growth and susceptibility to antibiotics. The second and third refer to antibiotic susceptibility. This thesis' hypotheses are founded on an analysis where changes in the extracellular environment are indirectly analyzed via differential gene expression, in addition to a study of phenotypic differences between cultures grown in the spaceflight environment and their matched ground controls. Extracellular mass transport is a slightly more complex biophysical issue that requires its further explanation.

2.5.1 Extracellular Mass Transport

A single cell's response to microgravity can be categorized under direct and indirect effects. Direct effects occur due to sensed acceleration signals or measureable weight, deformation (strain, bending, and torsion) or displacement of the cytoskeleton or cell organelles. On the other hand, indirect effects are those that occur as a result of prior changes that took place due to microgravity. Namely, bulk fluid phenomena, boundary layer-related effects and other mass transport phenomena (Klaus et al., 2004).

Similarly, mass transport can be categorized as active or passive in nature. Active transport entails stirring or pumping actions done either naturally, e.g. by flagella, or by human intervention. Passive transport can be categorized as gravity-dependent or –

independent. Density driven motion is an example of the first group while random, Fickian and osmotic diffusion are representative of the latter category. Density-driven convective currents can be produced by either concentration gradients or thermal gradients (Klaus et al., 2004).

During the last decades, mass transport phenomena have been proposed as the reason for multiple cellular observations made in space. As recent examples, three separate NASA Ames biosatellites have concluded that mass transport phenomena played an important role in changes in bacterial and fungal behavior.

First, the GeneSat automated satellite produced data that led scientists to hypothesize that the changes observed in growth curves were in part because nutrient delivery and waste removal from cells (mass transport phenomena) were potentially altered in microgravity (Kitts et al., 2007; Minelli et al., 2008; Ricco et al., 2007).

Second, it was concluded from the data produced by the O/OREOS nanosatellite with *B. Subtilis* on board that the bacteria grew more slowly in space than on Earth. It was stated that "the primary difference… is a change in mass transport of nutrients and waste products" (Nicholson et al., 2011).

Third, the PharmaSat team observed an extended lag phase on *S. cerevisiae* in microgravity compared to the ground controls. They also saw that the yeast grown in a medium with 4xMIC still presented metabolic activity in space. That team hypothesized that these changes occurred due to altered mass transport phenomena: nutrients were not reaching the cells as quickly and/or waste products were not being washed away as fast as in Earth since mass transport was a diffusion-only type of phenomenon (Kitts et al., 2009; Ricco et al., 2010).

Microgravity may affect cellular metabolism in part by altering mass transport processes governing nutrient uptake and waste removal. Klaus et al. (1997) proposed that

in microgravity, when the exponential growth phase was achieved, a pseudo-membrane of toxic metabolic byproducts would form (as an osmotic solute gradient) around the cell. This is similar to the 'deletion zone' hypothesized to promote improved protein crystallization in space (McPherson et al., 1999). On the other hand, in 1g when cell byproducts are less dense than the cell itself, they are washed away as the cell sediments. This also provides continued 'fresh' nutrients to the cell. When sedimenting, a cell reaches terminal velocity, where the forces of weight, buoyancy and shear are balanced; meaning that the cell feels its full weight as it sediments. Furthermore, fluid dynamics around a cell can be modeled with the momentum equation (derived from the Navier-Stokes equation) and the species concentration equation as in (Klaus et al., 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 BACTERIAL MODEL SELECTECTION

E. coli ATCC 4157 *Escherichia coli* (Migula) Castellani and Chalmers (ATCC® 4157™) (ATCC, 2012)) was chosen as the model species and strain for myriad reasons. *E. coli* is the bacterial research organism flown to space the most, as seen in Figure 1, which provides a wealth of data to compare against (Zea, Stodieck, & Klaus, 2014). Specifically, this strain has been flown on seven different space shuttle flights: STS-37, -43, -50, -54, -57, -60 and -62 (Klaus, 1994; Brown et al., 2002).

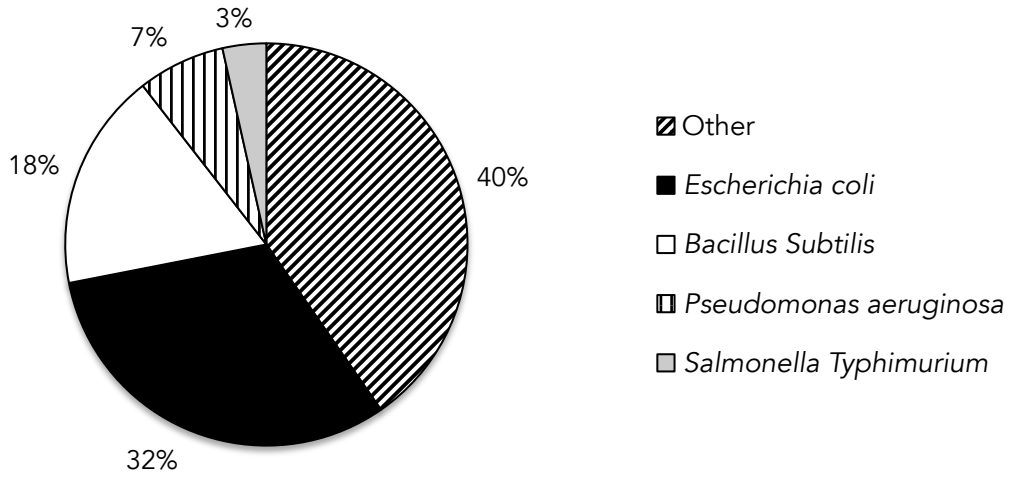


Figure 1. Distribution of bacterial species used on spaceflight experiments. *E. coli* has been the most commonly flown bacterial species to date (54 experiments), based on a study of over 171 space-based *in vitro* experiments (Zea et al., 2014).

This is a non-motile strain when grown with only glucose as the source of carbon. This is important because one theory suggests that motile cells could potentially disrupt the quiescent environment around it, which in turn may confound spaceflight results (Benoit & Klaus, 2007). As part of the human biome, *E. coli* is present in all human spaceflight and has been found on spacecraft surfaces and air (Novikova, 2004). It is an opportunistic pathogen, so it is likely to trigger infectious diseases during spaceflight (Ilyin, 2005), e.g. meningitis, invasive urinary tract infections, septicemia and diarrhea (Buchanan & Gibbons, 1975; Johnson, Gajewski, Lesse, & Russo, 2003). Finally, *E. coli* is an organism commonly used for other types of studies thus acquiring more knowledge about it may be beneficial to other parties.

3.2 GROWTH MEDIUM AND TEMPERATURE

Bacterial growth in a liquid medium in microgravity presents a low-shear stress environment similar to that sensed by bacteria in the human gastrointestinal, respiratory, and urogenital tracts (Nickerson, Ott, Wilson, Ramamurthy, & Pierson, 2004). *E. coli* was grown anaerobically in Medium E minimal medium as described in Vogel & Bonner (1956) supplemented with glucose (Fisher Scientific, Cat. No. D-16, Waltham, MA, USA) to a final 5 (g/L) concentration. The experiment was designed for bacteria to be cultured at 30°C, as this temperature allows for a clearer differentiation of the growth phases (Kacena, Merrell, et al., 1999). Actual temperature data show that the spaceflight samples of the experiment reported in Chapter 5 were maintained at 30.2°C ±0.7°C (four independent temperature data recorders) and the ground controls at 31.7°C ±0.4°C (another four independent temperature data recorders), i.e. there was only a 1.5°C average temperature difference, which makes no significant difference in growth based on pilot experiments. More details

presented in Appendix 3. The inoculum's cell density was 7.91×10^6 cell/mL (log-phase cells), which once diluted to the test starting conditions yielded a 1.22×10^6 cell/mL concentration.

3.3 CULTURE HARDWARE: FLUID PROCESSING APPARATUS (FPA)

The experiments took place in BioServe's Fluid Processing Apparatus (FPA), shown in Figure 2. The FPA is a spaceflight-rated glass barrel that permits to store up to a total of 6.5 mL of four different solutions and to mix them sequentially to initiate and terminate an experiment. Fluids are separated by rubber septa, which can be pushed to allow mixing through a bypass. FPAs were packed in groups of eight inside Group Activation Packs (GAP), which in turn were housed inside BioServe's Commercial Generic Bioprocessing Apparatus (CGBA) for temperature control (Hoehn, Klaus, & Stodieck, 2004).

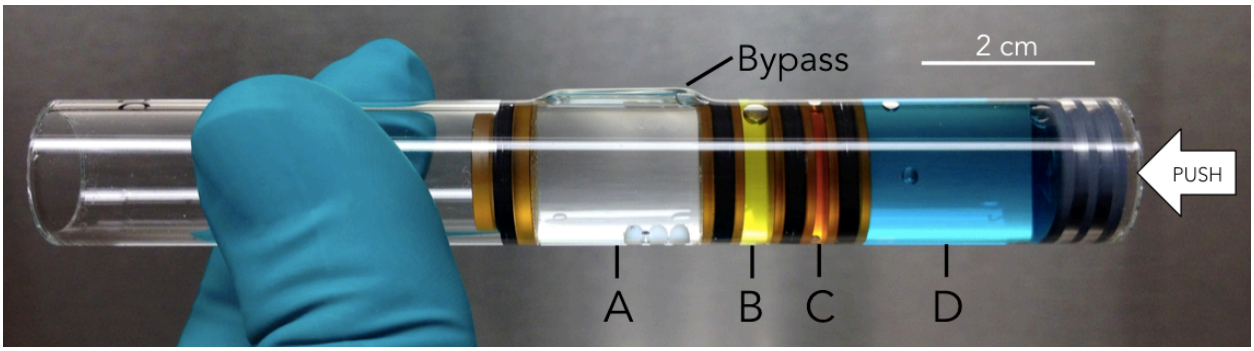


Figure 2. Fluid Processing Apparatus (FPA). BioServe's FPA loaded with colored solutions to best describe the actual contents per chamber of the AES-1 spaceflight experiment configuration: A – 2.75 mL of sterile growth medium with glucose; B – 0.50 mL of inoculum in growth medium; C – 0.25 mL of antibiotic solution; D – 2.10 mL of fixative (either paraformaldehyde or RNA Later II). In this figure, pushing from right to left would move the septum separating chambers A and B into the bypass, thus allowing for the solution in chamber B to be transferred and mixed into chamber A. The actual solutions as flown in AES-1 were all colorless.

3.4 ANTIBIOTICS

Two antibiotics were used. First, Gentamicin Sulfate (MP Biomedical, Cat No. 1676045, Santa Ana, CA, USA), an aminoglycoside that interrupts protein synthesis by

binding to the 30S subunit of the bacterial ribosome and which has been flown in experiments onboard STS-69 and STS-73 (Kacena, Merrell, et al., 1999), and the Soviet/Russian space station Mir (Juergensmeyer et al., 1999) (reported in the last reference simply as Gentamicin, with no further details). Seven different Gentamicin Sulfate solutions were prepared in distilled water and filter-sterilized (0.20 μm) for flight. Their concentrations varied so that, when introduced into the culture, they would range from 25 to 175 μg/mL. They were stored at 4°C until needed for loading the FPA. Second, Colistin Sulfate (Sigma-Aldrich, Cat No. C4461, Saint Louis, MO, USA), a polypeptide that kills bacteria by solubilizing its membrane and which has been used in experiments onboard Salyut 7 (Lapchine et al., 1986; Tixador, Richoilley, Gasset, Planel, et al., 1985; Tixador, Richoilley, Gasset, Templier, et al., 1985) and STS-61-A (Lapchine et al., 1986; Moatti et al., 1986) (reported in all of these references simply as Colistin, with no further details). Seven different Colistin Sulfate solutions were prepared in distilled water and filter-sterilized (0.20 μm) for flight. Their concentrations varied so that, when introduced into the culture, they would range from 1 to 7 μg/mL. They were stored in the dark (this drug at these low concentrations is light sensitive) at -20°C until needed for loading the FPAs.

3.5 BASELINE LOWEST ANTIBIOTIC CONCENTRATION

Drug activity against an organism and bacterial resistance are usually quantified via the drug's minimum inhibitory concentration (MIC). To allow for comparison through laboratories, MIC is determined through standardized protocols as described in (Andrews, 2001; BSAC, 2012). Spaceflight experimentation introduces special requirements on the hardware and by extension, to the experiment design. In other words, stringent compliance to standardized procedures used on Earth is not always possible when conducting space life

sciences experiments. In the case of this experiment, the procedure to define MIC had to be modified in order to accommodate hardware and operational limitations derived from spaceflight. The lowest antibiotic concentration was defined on Earth as the drug concentration needed to inhibit bacterial growth in an FPA with an *E.coli* culture of 1.0×10^7 cell/mL in Medium E supplemented with 5 g/L glucose for 32 hours at 30°C (i.e. as close to flight conditions as possible). These lowest antibiotic concentrations were identified as 25 μg/mL and 1 μg/mL for Gentamicin Sulfate and Colistin Sulfate, respectively. The concentrations flown in the experiment described in Chapter 5 were therefore multiples of these concentrations from 1x up to 7x.

3.6 FIXATIVE

To allow for post-flight analyses, samples were fixed in paraformaldehyde (PFA) for the phenotypic studies and in RNA Later II for the genotypic assays. Paraformaldehyde (ACROS, Cat. No. 41678, New Jersey, USA) solutions in PBS (Fisher Scientific, Cat. No. TA-125-PB, Waltham, MA, USA) were prepared (pH 7.0) and filter sterilized (0.20 μm) so that, when mixed with the cultures, would yield a 1.5% concentration. RNA Later II (Life Technologies, Cat No. B7024, Carlsbad, CA, USA) was used for the gene expression analysis samples.

3.7 OPTICAL DENSITY AND CELL CONCENTRATION COUNT

All of the analyses were conducted in labs post-flight after sample return to Earth. Optical Density (OD) measurements of the 72 flight and 109 FPAs fixed with PFA were acquired with a Fisher Scientific Multiskan™ FC Microplate Photometer at 600 nm. Three individual samples were taken from each FPA, thus totaling over 500 OD data points. To count for potential changes in cell sizes corrupting the optical density data, actual cell

counts using a hemacytometer (as described in Appendix 1) were conducted on each of the 181 samples.

3.8 ANALSYS OF CELL MORPHOLOGY: PHASE CONTRAST AND TRANSMISSION ELECTRON MICROSCOPY (TEM)

Since the samples were already fixed in 1.5% PFA, no sample preparation was required for phased contrast microscopy, which was used for analyzing cell and colony morphology, and cell length and diameter. Phase contrast microscopy was performed using a Carl Zeiss Axio Imager M2 and a Nikon E600 Widefield Microscope. Cell length and diameter (624 data points) were acquired using ZEN (Zeiss, 2014) and FIJI (LOCI, 2014) software.

Originally, cells were stained with FM4-64 lipophilic styryl dye (Molecular Probes Cat No. T-3166) as in (Lewenza, Vidal-Ingigliardi, & Pugsley, 2006; Pohl et al., 2007), and DAPI (4',6-Diamidino-2-phenylindole, Sigma-Aldrich Cat No. D9542), to contrast the cell envelope from the nucleotides, respectively. The use of these two dyes together improves visualization of *E. coli* cell membrane (Fishov & Woldringh, 1999). After staining, cells were observed utilizing a Zeiss 510 confocal laser scanning microscope. This microscope is capable to use a pinhole to reject light that comes from outside the focus area. This permits to make “optical sections” of bacteria. However, phase contrast microscopy proved ideal for measuring cell size, as the cell envelope was clear from the background and it required no staining. Cell surface was calculated from the length and diameter data and by modeling a bacterial cell as a rectangular cylinder with two hemispheres as seen in Figure 3.

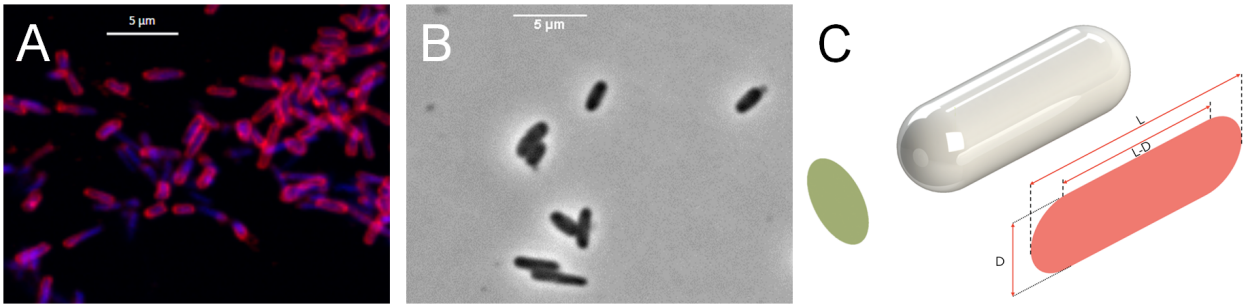


Figure 3. Cell size measurement. Originally, cell size measurements were going to be acquired dying the cells with FM4-64 and DAPI stains and observing them with fluorescence microscopy, as shown in image (A) – image taken with a Zeiss 510 confocal laser scanning microscope. However, it was more convenient to acquire these measurements via phase contrast microscopy (B) – image taken with a Nikon E600 Widefield Microscope. (C) A bacterial cell was modeled as a rectangular cylinder with two hemispheres of total length L and diameter D, as measured on the microscope images. This capsule was composed of a cylinder of length L-D and two hemispheres of diameter D. The minimal cross section area is shown as the green, circular projection, and the maximum as the red projection.

Cell surface area was calculated per the following equation:

$$A = \pi D(L - D) + \pi D^2$$

where A indicates area, D represents diameter, and L total cell length. The surface area was calculated for each of the 312 cells measured and error bars were based on the overall data. Stokes radii were calculated from the volume of each cell, assuming a spherical shape. Student's t-test analysis was conducted to determine statistical significance of the different parameters assessed.

For Transmission Electron Microscopy (TEM), conducted at the Core Facility for Integrated Microscopy at the University of Copenhagen, samples were fixed with 2% v/v glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2). Following centrifugation the supernatant was replaced and the sample pellets re-suspended and rinsed in 0.15 M sodium cacodylate buffer (pH 7.2) three times. Next, the sample pellets were embedded in

low-melting point agarose and postfixed in 1% w/v OsO4 in 0.12 M sodium cacodylate buffer (pH 7.2) for 2 hours. The specimens were dehydrated in graded series of ethanol, transferred to propylene oxide and embedded in Epon according to standard procedures. Sections, approximately 80 nm thick, were cut with a Reichert-Jung Ultracut E microtome and collected on copper grids with Formvar supporting membranes, stained with uranyl acetate and lead citrate, and subsequently examined with a Philips CM 100 TEM (Philips, Eindhoven, The Netherlands), operated at an accelerating voltage of 80 kV and equipped with an OSIS Veleta digital slow scan 2k x 2k CCD camera (Olympus, Germany). Digital images were recorded with the ITEM software package.

CHAPTER 4

IN-VITRO ANTIBIOTIC ACTIVITY AND BACTERIAL CELLULAR ENVELOPE INVESTIGATIONS IN SPACE

Spaceflight offers a unique platform for conducting research on antibiotic effectiveness and bacterial resistance to antimicrobials. The applications of these types of investigations may not only positively impact future human space exploration, but may also help improve human health on Earth. A comprehensive review of over 400 publications and international databases was conducted to characterize the current state of knowledge on spaceflight antibiotic activity and bacterial resistance, and to identify potential next research steps in these fields. Experiments conducted in free-flying satellites, human spacecraft and space stations, and even onboard orbiting rocket stages, were considered, together with their testing details, findings, and conclusions. From this investigation, only six different spaceflight experiments were identified to interrogate antibiotic activity in space (Tixador et al., 1985; Moatti et al., 1986; Lapchine et al., 1986; Tixador et al., 1994; Gasset et al., 1994; Klaus, 1994; Kacena & Todd, 1999; Juergensmeyer et al., 1999). The analysis of their methodologies and results suggests that cell envelope changes observed on bacteria during spaceflight may be related to antibiotic effectiveness. Therefore, this review also includes the four experiments where changes on cell envelope were assessed (Tixador

et al., 1985; Menningham & Heise, 1994; Tixador et al., 1994; Gasset et al., 1994; Juergensmeyer et al., 1999).

Preceding human spaceflight, the Soviets and Americans launched missions containing, among other organisms, bacteria. The first Soviet and American satellites to carry bacterial samples, Korabl-Sputnik 2 (incorrectly called "Sputnik 5" in the West due to lack of knowledge) and Discoverer 17, both in 1960, helped scientists verify if the cells would survive the exposure to the space environment (Bulban, 1961; Zhukov-Verezhnikov et al., 1962). Bacterial experiments' focus during the 1960's and 1970's revolved around viability, growth, and lysogeny – or the reproduction of bacteriophages, small viruses that infect bacteria (Zea et al., 2014). These investigations suggested that bacterial growth was increased in space, meaning that spaceflight samples usually had higher final number of cells than their matched ground controls.

Two experiments, one conducted onboard Skylab and another during the Apollo-Soyuz Test Project (ASTP), investigated if bacteria exposed to the spaceflight environment showed any differences to antibiotic susceptibility *after* their return to Earth. They had conflicting results: the Skylab experiment concluded that bacteria were more *susceptible* to antibiotics after being exposed to the space environment (Floyd, 1974; Summerlin, 1977), while the ASTP research project reported that antibiotic *resistance* was increased in bacteria sent to space (Taylor & Zaloguev, 1978).

This was the knowledge landscape at the beginning of the eighties when the first experiment assessing antibiotic activity in space, Cytos 2, took place onboard the Soviet space station Salyut 7. This research project aimed at answering two new scientific questions that became the foundation for current investigations: 1) "Are there changes in antibiotic activity in space?" and 2) "Are there changes in bacterial cell envelope thickness in space?"

4.1 ASSESSMENT OF THE METHODOLOGIES USED ON SPACE-BASED INVESTIGATIONS

4.1.1 Limitations of Experiments Conducted in Space

Space-based research adds a series of challenges to scientists that are usually not considered for Earth-bound experiments. Media, buffers, and all solutions and materials need to be approved for launch; the experiments may need to be handed over and integrated into the launch vehicle well in advance of lift off, and it may take additional time for the investigation to start after reaching space. This generally poses a hindrance to time-sensitive life science investigations. These issues may be exacerbated if the experiment is taking place in a human-tended space station, as safety regulations are increased and astronaut time may not be readily available. Furthermore, what could be considered standard laboratory equipment on the ground may not be available in space and engineers and payload integrators are needed to ensure proper functionality of the experiment hardware. While a scientist may open a petri dish to assess or manipulate a culture on Earth, in space "levels of containment" must be maintained in between the sample and the environment – their number depending on the bacterial biosafety level.

These and other issues require that established and standardized protocols be updated and customized for spaceflight. An example of this is the identification of a drug's Minimum Inhibitory Concentration (MIC), or the lowest concentration needed to inhibit bacterial growth. Although MIC protocols are well defined in the U.S. and Europe (Andrews, 2001; BSAC, 2012), these may not be easily implemented. This, and the use of different hardware, makes direct comparison in between spaceflight experiments difficult.

Finally, one of the biggest challenges for researchers to conduct experiments in space is up- and down-mass, or bringing equipment and samples to and from space. The International Space Station (ISS) has partly alleviated these problems by providing an equipped orbiting laboratory regularly tended by servicing spacecraft. Although the problem still exists, scientist can now send experiments with large enough sample sets to help them achieve statistical significance in their studies. This was harder to accomplish during the first decades of space-based research, where up-mass and astronaut time was even scarcer – only the latest three experiments analyzed in this review report statistical significance on their results.

4.1.2 Potential Re-Adaptation to 1*g*

Fixing the samples *after* return to Earth or not fixing them at all introduces a confounding factor: potential re-adaptation to 1*g*. For example, some of the bacterial cells flown to the Soviet space station Salyut 7 were challenged with antibiotics in space and some were brought back to Earth for post-flight analysis. This study concluded that increased resistance disappeared after return to Earth (Tixador, Richoilley, Gasset, Planel, et al., 1985); in other words, characters such as increased resistance in space were not acquired and may be lost at return to 1*g*. Thus, if antibiotic effectiveness or cell envelope thickness measurements (or any other, for that matter) are not taken *in-situ*, or if they are acquired on samples fixed *after* return to 1*g*, re-adaption to 1*g* may confound the results. The fixation of samples in space introduces one more step that increases hardware and experimental complexity, and was rare during the first decades of space life sciences research. From the six antibiotic activity investigations, only three report acquiring their data *in situ*, or fixing the samples prior to return to Earth for post-flight analysis (Tixador

et al., 1985; Klaus, 1994, Kacena & Todd, 1999). Similarly, from the four cell envelope investigations, only one documents in-space fixing taking place (Tixador et al., 1994).

4.1.3 Growth Medium

Four of the six antibiotic activity studies (Cytos 2, Antibio, Antibio 23F, and STS-57/60) cultured the bacteria in a liquid medium (Tixador et al., 1985, Moatti et al., 1986; Tixador et al., 1994; Klaus, 1994). The other two (STS-69/73 and the one onboard Mir) grew them in agar, since part of their objectives was to interrogate the role of medium (liquid/solid) on drug effectiveness (Kacena & Todd, 1999; Juergensmeyer et al., 1999). MIC identification protocols on liquid medium on Earth are based on the introduction of antibiotics during the acceleration phase (in between lag and exponential growth phases) of bacterial growth. However, this introduces further complexity to space-based experiments such as additional astronaut operational steps, or extra pumps and valves. Thus, all of the spaceflight experiments were antibiotic activity was interrogated had the antibiotic already in the growth medium, except for the experiment conducted onboard the Mir space station. On that experiment, antibiotic effectiveness was tested *after* the experiment returned to Earth by placing antibiotic discs on the agar on subcultures grown from the samples flown to space (Juergensmeyer et al., 1999). Three of the four experiments that assessed changes on bacterial cell envelope were conducted on liquid medium, the exception being, again, the Mir experiment (Tixador et al., 1985; Tixador et al., 1994; Menningham & Heise, 1994; Juergensmeyer et al., 1999).

4.1.4 Motility

Although most spaceflight studies have reported the same general finding of an increase in bacterial populations in space with respect to ground controls, there have been a

few exceptions that noted no differences. A literature review published in 2007 found a trend relating cell motility with experimental outcome where the studies that reported exceptions to the general finding tended to used motile strains (Benoit & Klaus, 2007). It was hypothesized that the bacterial flagella disrupted the quiescent medium around the cell, which is one of the hallmarks of bacterial growth in liquid media in microgravity. However, the importance of motility had not been systematically characterized but until 2007, so it was not a generally recognized bacterial selection criterion before then.

One of the bacterial strains employed in two of these investigations – *E. coli* K12 ATCC®25922™ used on the Antibio and Antibio23F experiments – was motile (Benoit & Klaus, 2007); one was non-motile – *E. coli*®ATCC 4157™ used on the STS-57/-60 and STS-69/-73 investigations – and the motility of the others strains is unknown (see and **Error! Reference source not found.** at the end of this chapter, for details).

4.2 REVIEW OF THE RESULTS

4.2.1 Changes in Antibiotic Activity *in-vitro* in Space

In-vitro bacterial proliferation in antibiotic concentrations higher than those needed to inhibit growth on Earth has been observed since the first antibiotic activity experiment, Cytos 2; however, conflicting results have been published. It has been observed in space that *E. coli* collected from an astronaut's microflora was capable of proliferating in 4XMIC of Colistin (polymyxin), 4XMIC of Kanamycin (aminoglycoside) (Tixador, Richoilley, Gasset, Planel, et al., 1985); that *E. coli* K12 ATCC®25922™ thrived in 2XMIC of Colistin (Lapchine et al., 1986; Moatti et al., 1986); and *E. coli* ATCC®4157™ grew in 1X MIC Gentamicin (aminoglycoside) (Klaus, 1994). Additionally, a bacteriostatic experiment with *E. coli* and Colistin showed a 100X increase in final cell count in space with respect to

ground controls (Lapchine et al., 1986; Moatti et al., 1986). Based in the difficulties stated above, statistical significance was not reported in any of these results, with the exception of (Klaus, 1994).

No differences in MIC between spaceflight and ground control samples were reported in *Staph. aureus* challenged with Oxacilin (penicillin), Chloramphenicol (cholarmphenicol), and Erythromycin (macrolide) (Tixador, Richoilley, Gasset, Planel, et al., 1985); nor in *E. coli* ATCC®4157™ challenged with Colistin (Klaus, 1994). No changes were observed in final cell count when *E. coli* was challenged with dihydrostreptomycin (Tixador et al., 1994). In this study, antibiotic binding was also quantified via radioactivity tritium-labeled dihydrostreptomycin and the results suggested that there was a slower antibiotic uptake in space (Tixador et al., 1994).

Finally, an increase in antibiotic *effectiveness* has only been reported on the STS-69/-73 experiment – where a 10% increase in Gentamicin effectiveness was observed on non-motile *E. Coli* grown in agar (where the cell doesn't experience as many changes on the extracellular environment in space with respect to 1g, as it would on liquid medium, as explained in section 4.3.2 The Role of Fluid Behavior) – and on some of the tests of the Mir experiment (Kacena & Todd, 1999; Juergensmeyer et al., 1999). On the latter, after the return of the unfixed samples to Earth, subcultures of the four flown species were grown and challenged with 12 different drugs. They concluded that *after* spaceflight, bacteria became more susceptible in seven cases and more resistant in two with respect to pre-flight values – including *E. coli* becoming more resistant to Penicillin (Juergensmeyer et al., 1999).

Klaus (1994) concluded that antibiotic effectiveness tended to decrease on the drugs to which *E. coli* could adapt, to which Juergenesmeyer et al. (1999) agreed and added "*E. coli* tended to become more susceptible to the antibiotics to which it was clinically

susceptible on the ground, and more resistant to those antibiotics to which it was clinically resistant on the ground". They did not see this pattern on the other three tested bacterial species.

4.2.2 Changes in Cellular Envelope in Space

Cytos-2 was not only the first experiment to assess antibiotic activity in space but it also included a separate cellular envelope thickness investigation, concluding that although there were no changes on *E. coli*, there were on *Staph. aureus* (Tixador, Richoilley, Gasset, Planel, et al., 1985). Although no statistical analyses are indicated, electron microscopy images of ground and spaceflight samples are presented in (Tixador, Richoilley, Gasset, Planel, et al., 1985; Zaloguyev et al., 1984). Zaloguyev et al. (1984) indicated that *Staph. aureus* cell envelope measured 28nm on the ground samples while it was 89nm on the spaceflight cultures. An English translation of the abstract was published in (NASA, 1985). Three other experiments have followed suit and all of them reported that there were no changes in cell envelope: either on *E. coli* (Gasset et al., 1994; Tixador et al., 1994), on *B. subtilis* (Menningmann & Heise, 1994) nor in cell structure in general on *E. coli, B. subtilis,* or *Staph. aureus* (Juergensmeyer et al., 1999). Neither of these publications state achieving statistical significance in their analyses, and from all four investigations only (Gasset et al., 1994; Tixador et al., 1994) report having fixed the samples in space.

4.3 DISCUSSION

As seen in Figure 4, the Cytos 2 experiment aimed at answering two questions: Q1 "Are there changes in antibiotic activity in space?" and Q2 "Are there changes in bacterial cell envelope in space". Based on their observations that *E. coli* could proliferate in four times the concentration of antibiotic compared to ground controls, and that there was an

increase in cellular envelope thickness on *Staph. aureus* in space, they proposed two hypotheses. H1: thicker cellular envelope translates into reduction of antibiotic introduction speed; and H2: increased bacterial multiplication stimulates antibiotic resistance.

4.3.1 Gravity *vs.* Cosmic Radiation

To further investigate the first question and the first hypothesis, the same research group conducted two more experiments: Antibio in 1985 and Antibio23F in 1992. The first experiment showed that *E. coli* in space could grow in concentrations twice as high as those that it could survive on Earth, while the second showed no differences in that regard. Each of these experiments had two sets of cultures in space: one under "normal spaceflight microgravity" and another in a 1*g* centrifuge on orbit. Bacteria behaved similarly in each of these two sets, on both flights. These results suggested that there was another independent variable, other than gravity, associated with spaceflight being responsible for their observations. Therefore, this team proposed a new hypothesis H3: the differences observed between spaceflight and ground controls are due to cosmic radiation and not because of gravity. However, both of these experiments used *E. coli* ATCC®25922™ as their model organism – a motile strain – and it is not clear what role this parameter may have played on their results and conclusions. On the same Space Shuttle mission where Antibio23F was conducted (STS-42), another experiment termed "Spores" took place in which a set of cultures were also placed in a 1*g* centrifuge in orbit. In this experiment, the samples placed in the centrifuge behaved similarly to the ground controls and not to the microgravity set. This team then put forward a counter-hypothesis H4: the differences observed between spaceflight and ground controls are due to gravity and not because of cosmic radiation. Menningham and Heise (1994) did not report the strain of *B. subtilis* they used on the Spores experiment. To acquire data to interrogate the role of these two independent

variables – gravity and radiation – new experiments should consider using a 1*g* centrifuge in orbit, and using non-motile strains of bacteria.

4.3.2 The Role of Fluid Behavior

Another experiment was conducted onboard Space Shuttle flights STS-57 and -60, in which (non-motile) *E. coli* ATCC®4157™ was observed to proliferate in Gentamicin concentrations that were inhibitory on Earth (Klaus, 1994). This investigation assessed other parameters beyond antibiotic effectiveness and concluded that there was a shorter lag phase and a higher final bacterial cell count in space compared to ground controls. A new hypothesis was proposed from this investigation H5: changes in net extracellular mass transport of antibiotics expose the bacteria to lower doses, allowing them to develop resistance. From this, a new question was posed Q4: Does fluid behavior play a role on bacterial susceptibility to antibiotics in space? To answer this, the next investigation – conducted onboard Space Shuttle flights STS-69 and -73 – cultured bacteria in agar. The same (non-motile) *E. coli* strain (ATCC®4157™) as the previous flight was challenged with Gentamicin. Just as with previous experiments where bacteria was cultured in liquid medium, it was concluded from this experiment that bacterial growth was increased in space with respect to ground controls. However, antibiotic *effectiveness* was observed to increase in a 10% in space in this agar-based test. On the following experiment, conducted onboard space station Mir, bacteria was cultured in agar in the microgravity environment for four months, not only trying to assess question Q4 but also a new one Q5: Does the duration of exposure to spaceflight affect susceptibility to antibiotics? This experiment yielded mixed results, were antibiotic efficacy was observed to increase and decrease, depending on the bacterial species and antibiotic tested. However, it must be kept in mind that in this experiment, it was not the spaceflight samples that were challenged with drugs,

but subcultures grown from those that flew, with at least 10 hours (i.e. several generations downstream) in between sample return to Earth and experiment handover to the scientists (Juergensmeyer et al., 1999).

Changes in extracellular transport in liquid medium is to date the hypothesized reason behind several bacteria-related spaceflight phenomena, including modified growth behavior, and increased virulence (Benoit et al., 2008; Benoit & Klaus, 2007; Kitts et al., 2009; Klaus, 2004; Klaus et al., 2004, 1997; Klaus & Howard, 2006; Nicholson et al., 2011; Ricco et al., 2007, 2010; Todd & Klaus, 1996). In order to systematically test this hypothesis, new experiments should consider culturing bacteria in liquid medium and agar in a synchronous fashion.

4.3.3 Relationship between Cellular Envelope and Antibiotic Activity

Results from the four investigations on cellular envelope changes due to spaceflight are inconclusive. On one hand, cellular envelope thickness measurements of 28nm and 89nm were reported on the ground controls and spaceflight samples of Cytos-2, respectively. However, no statistical analysis could be found and these samples were not fixed in space. Furthermore, temperature tracking in between sample return to Earth in Kazakhstan, and fixation in glutaraldehyde in Moscow was not possible (Lapchine et al., 1986).

On the other hand, the next three investigations reported no changes on cellular envelope between samples cultured in space and matched ground controls. However, in only one of these three were samples fixed in space, Antibio23F, the experiment that used a motile strain of *E. coli*. For another one of these three investigations, Spores, no statistical analysis could be found. The other experiment that reported no changes in cellular envelope conducted the assessment not on spaceflight samples but on subcultures grown on Earth.

Because of these inconsistencies, cellular envelope investigations should be continued as it has been proved that changes on the bacterial cell envelope are correlated with antibiotic resistance here on Earth (Sieradzki & Tomasz, 2003), and because conclusive data hasn't yet been produced. Finally, it is recommended that standardized protocols for conducting these types of analyses be used, if possible, to make the spaceflight results available to other investigations on Earth.

4.3.4 Statistical Significance and Potential Re-Adaption to 1*g*

Maintaining a stringent and critical approach, and disregarding the difficulties of space-based experimentation – especially during the first few decades of spaceflight – only the results where statistical significance was achieved and reported should be considered. Furthermore, the interpretation of the results that may have been confounded by potential re-adaptation to 1*g* should be questioned. Under these strict criteria, the following results may be considered statistically valid and free from potential re-adaptation to 1*g*:

1. Non-motile *E.coli* ATCC®4157™ proliferated in liquid medium in 1XMIC of Gentamicin while no growth was observed on matched ground controls (Klaus, 1994).
2. Antibiotic-free growth of non-motile *E. coli* ATCC®4157™ has been observed to increase in spaceflight with respect to ground controls, in liquid medium (Klaus, 1994) as well as in agar (Kacena & Todd, 1999).
3. Non-motile *E. coli* ATCC®4157™ challenged with Gentamicin in agar, showed a 10% increase in drug effectiveness in space than compared to 1*g* (Kacena & Todd, 1999).

4.4 RECOMMENDATIONS FOR FUTURE RESEARCH

Spaceflight places challenges on research that are best addressed during early experiment design. It is recommended to use low-biosafety level organisms and, depending on the scientific objectives, to keep bacterial motility as a strain selection criterion.

Similarly, it is suggested to use non-toxic, and non-hazardous materials to the extent possible. The experiment should be planned to minimize the impact of the delay between sample preparation and experiment start (due to payload integration into the spacecraft/launch vehicle, potential launch delays, initiation of operations after reaching space, etc.). This can be achieved by maintaining the organisms in stasis, either by temperature (if power and temperature regulation are available to the organism habitat), or by maintaining it in a medium without a source of glucose or metabolic energy, if possible. Given the limitation on up-mass, it is also recommended to prioritize sample replicate number over amount of testing conditions to enable statistically significant results.

Ideally, scientific data should be acquired in space; however, this is often difficult to achieve. To avoid potential re-adaption of the organism to 1*g*, the next best solution is to fix the samples in space as soon as the experiment has been completed. Researchers should also keep in mind what assays and protocols will be conducted on Earth and choose their fixative accordingly.

The two original questions posed before Cytos 2 still need answering; additionally, new ones have been presented. The role of gravity *vs.* cosmic radiation, of fluid behavior, and of bacterial motility on the observed results has been disputed. To address these variables in a systematic fashion, researchers should consider the use of a 1*g* centrifuge in orbit, the medium used (liquid *vs.* agar), and the selection of motile or non-motile strains of bacteria. Other aspects, such as the role of growth medium constituents (e.g. phosphates) should also be examined.

It is encouraged that cellular envelope investigations are continued, as it has been proved that changes on the bacterial cell envelope are correlated with antibiotic resistance here on Earth (Sieradzki & Tomasz, 2003), and because conclusive data hasn't yet been

produced regarding cell envelope changes during spaceflight. Finally, -omics types of analyses, e.g. transcriptomics and genomics, and the use of standardized protocols are recommended to make the spaceflight results useful and compatible to other investigations on Earth.

Table 2. Bacterial information, independent variables and data acquisition methodologies.

Launch Year	Experiment	Species	Strain	Motility	Starting cell cc	Variables measured	Data Acquisition Methodologies	Reference
1982	Cytos 2	*E. coli*	astronaut microflora	NR	2.5x10^5 cell/mL	Growth. Antibiotic effectiveness. Cell envelope thickness	Antibiotic efficacy: in-flight MIC (24 hours)-visual observation due to red phenol pH marker. Cell envelope thickness: fixed post-flight for Electron Microscope	(Tixador et al., 1985 A) (Tixador et al., 1985 B) (Lapchine et al., 1986)
		Staph. aureus	astronaut microflora	NR	2.5x10^5 cell/mL			
1985	Antibio	*E. coli*	K12 ATCC 25922	Motile (Benoit and Klaus, 2007)	2.5x10^5 cell/mL	Antibiotic effectiveness	MIC and cell count at bacteriostatic concentration	(Moatti et al., 1986) (Lapchine et al., 1986) (Lapchine et al., 1987)
1992	Spores	*B. subtilis*	NR	NR	NR	Growth rate. Frequency of sporulation. Re-adaptation to 1g. Repair capacity. Cell wall structure	Growth: in-flight photometer. Cell wall: post-flight electron microscopy	(Menningham and Heise, 1994)
1992	Antibio23F	*E. coli*	K12 MC4100 RelA1+	NR	4.5x10^4 cell/mL	Growth, viability. Cell size. Cell shape. Cell envelope thickness	All samples were placed at 5C at experiment end, except for those used for cell envelope thickness, which were fixed in space. Post-flight analysis: Growth: electronic particle counter (MC4100 strain) or spectrophotometer 530nm (25922 strain); cell viability by "spiral inoculator" and CFU/mL; antibiotic binding by radioactivity and cell count; cell ultrastructure on electron microscopy	(Gasset et al., 1994)
			ATCC 25922	Motile (Benoit and Klaus, 2007)	5x10^4 cell/mL	Growth. Cell ultrastructure. Antibiotic activity. Cell envelope thickness		(Tixador et al., 1994)
1993	STS-57/-60	*E. coli*	ATCC 4157	Non-motile	1.0x10^6 (inoculum)	Growth. Antibiotic effectiveness	Samples fixed in space. Growth and antibiotic effectiveness by cell count using a hemacytometer	Klaus' thesis, 1994 (Chapter 6)
1995	STS-69/-73	*E. coli*	ATCC 4157	Non-motile	5x10^5 cell/mL	Growth. Antibiotic effectiveness	Samples fixed in space. Cells were removed from agar using non-lytic surfactant. Growth and antibiotic effectiveness by cell count using a hemacytometer	(Kacena and Todd, 1999)
1997	Mir	*B. subtilis*	ATCC 6051	NR	1x10^8 cell/mL in nutrient broth prior to placing on agar	Antibiotic effectiveness. Cell structure	Assessments were not conducted on the spaceflight cultures but post-flight on subcultures. There were 10 hours in between return to Earth and experiment handover. Antibiotic effectiveness using antibiotic disks. Cell structure via gram staining and electron microscopy	(Jurgensmeyer et al., 1999)
		E. coli	ATCC 10798					
		Staph. aureus	ATCC 6538P					
		Pseudomonas aeruginosa	ATCC 27853					

Table 3. Materials, experimental conditions and hardware.

Experiment	Medium	Carbon source	Antibiotic tested	Fixative	Temp	Experiment Duration	Growth Chamber Vol.	Hardware
Cytos 2	API 10 M	NR	Colistin, Kanamycin Oxacilin, Chloramphenicol, Erythromycin	At experiment end: placed at 4C. After return to Earth: glutaraldehyde	37°C	24 hours from incubation to last MIC reading	1.2 mL	"Plastic bags" containing a glass ampule with the inoculum
Antibio	Undefined Medium (described in Moatti et al., 1986)	5 g/L C6H12O6	Colistin	NR	37°C	24 hours	0.7 mL	"Plastic bags" containing a glass ampule with the inoculum in Biorack
Spores	Nutrient Broth	NR	N/A	NR	NR	NR	NR	Type II Experimental Units (EU) I and II
Antibio23F	M9	0.4% w/v glucose	N/A	None for growth, viability, or antibiotic activity. For ultrastructure (EM): glutaraldehyde	37°C	9 hours	0.7 mL	"Plastic bags" in ESA Type I container in Biorack
	Peptone Medium	NR	Dihydrostreptomycin (tritiated and non-tritiated)			9 hours	0.7 mL	"Plastic bags" in ESA Type I container in Biorack
STS-57/-60	Medium E	5 g/L glucose	Polymyxin-E, Erythromycin, Gentamicin, Nalidixic acid, Actinomycin D, Colistin	ethanol	25°C flight, 19°C ground	From 1 to 10 days	2-5mL	BioProcessing Module (3 syringes and one valve)
STS-69/-73	Agar	5 g/L glucose	Gentamycin	2% v/v glutaraldehyde	37°C	27 hours (samples fixed at mid-log phase)	NR	Fluid Processing Apparatus (FPA)
Mir	Agar	NR	Ampicilin, Penicillin G, Cephalothin, Gentamycin, Kanamycin, Streptomycin, Tetracycline, Chloramphenicol, Erythromycin, Colistin, polymyxin B, Rifampicin	None	Mir "room temperature" (up to 32C)	4 months	1.5mL	Fluid Processing Apparatus (FPA)

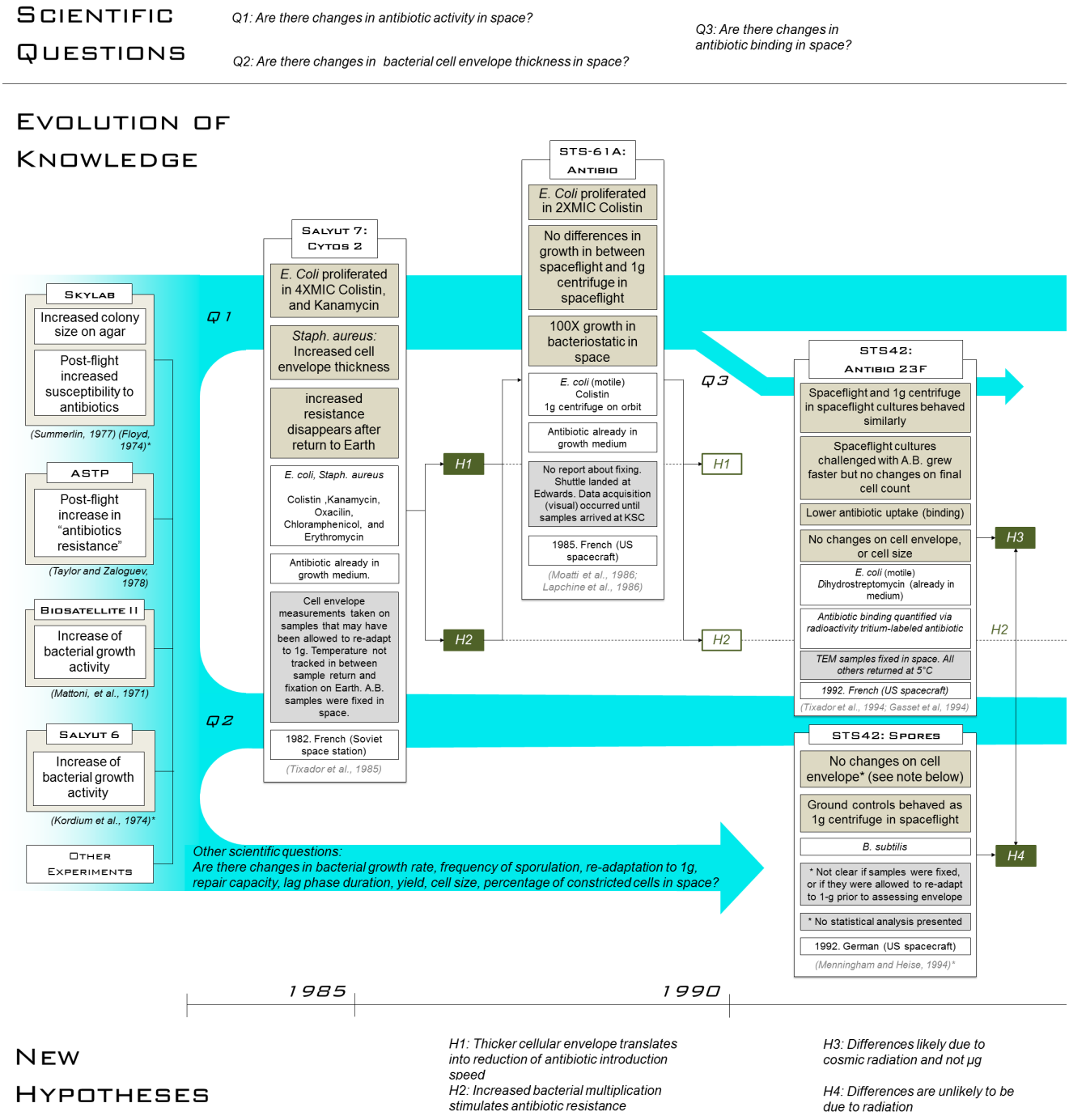
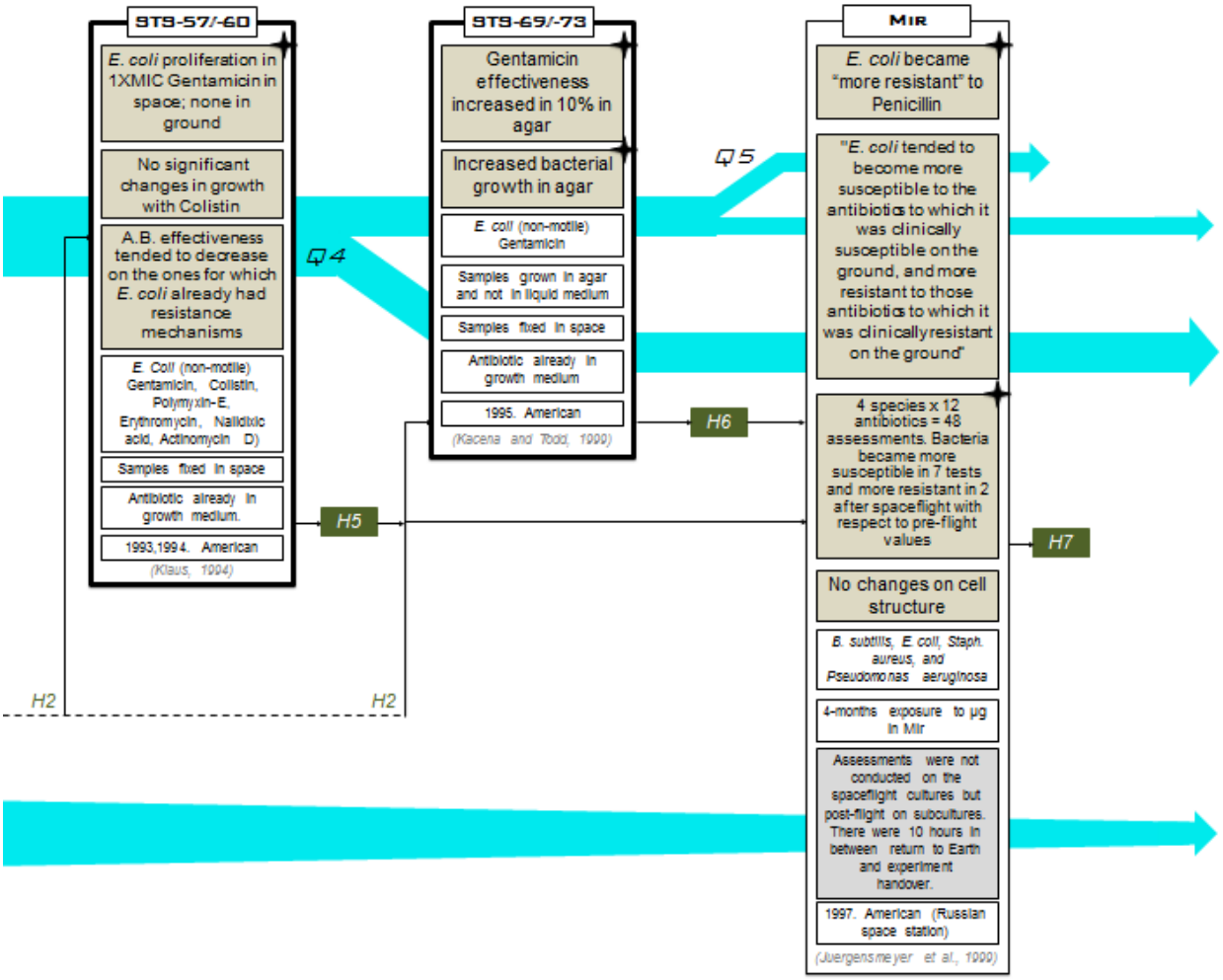


Figure 4. Evolution of knowledge on antibiotic activity and cellular envelope changes in space. Missions on a thick line box indicate that samples were fixed in space and therefore were not potentially compromised by re-adaptation to 1*g* and present statistical analyses on the results. A star on the upper-right corner of a box indicates a statistically-significant result. Boxes in tan represent results. Boxes in light gray represent a potential confounding factor.

Q4: Does fluid behavior play a role on bacterial susceptibility to antibiotics in space?

Q5: Does the duration of exposure to spaceflight affect susceptibility to antibiotics?



H5: Changes in net extracellular mass transport of antibiotics expose the bacteria to lower doses, allowing them to develop resistance

H6: Reduced antibiotic effectiveness is due to fluid behavior

H7: Increased resistance "is not as pronounced" on longer (4 months) missions

CHAPTER 5

OBSERVED PHENOTYPIC CHANGES IN *E. COLI* CHALLENGED WITH ANTIBIOTICS IN SPACEFLIGHT

Bacterial susceptibility to antibiotics has been shown *in vitro* to be reduced during spaceflight; however, the underlying mechanisms responsible for this outcome are not fully understood. In particular, it is not yet clear whether this observed response is due to increased drug resistance (a microbial defense response) or decreased drug efficacy (a microgravity biophysical mass transport effect). To gain insight into the differentiation between these two phenomena, an investigation was undertaken onboard the International Space Station (ISS) in 2014 – Antibiotic Effectiveness in Space-1 (AES-1). For this purpose, *E. coli* was challenged with two antibiotics, Gentamicin Sulfate and Colistin Sulfate, at concentrations higher than those capable of inhibiting growth on Earth. Phenotypic parameters (cell size, cell envelope thickness, population density and lag phase duration) and gene expression were compared between the spaceflight samples and ground controls cultured in varying levels of drug concentration.

5.1 MATERIALS AND METHODS

Most of the materials and methods behind AES-1 are explained in Chapter 3. However, some details are specific to the spaceflight experiment and here described.

5.1.1 Sample Preparation and Loading

All of the hardware items were autoclaved. Two sterile PTFE mixing balls were introduced together with 2.75 mL of sterile Medium E with 5.91 g/L glucose (to yield a final 5 g/L concentration when mixed with the inoculum) into the A chamber of each FPA. The FPAs were then incubated for 48 hours at 37ºC for contamination check. Next, chamber B was loaded with 0.50 of inoculum (at 7.90×10^6 cell/mL to yield a 1.22×10^6 cell/mL concentration when mixed with the growth medium) in glucose-free Medium E. Chamber C was then loaded with 0.25 mL of antibiotic solution as necessary for each experimental condition. Finally, the corresponding fixative was introduced into Chamber D (2.10 mL). The FPAs were stored at 4°C and transported to NASA Wallops in Virginia. There, RNA Later II solution was re-homogenized since this fixative tends to form crystals at lower temperatures. The FPAs were then loaded into GAPs and these, in turn, into CGBA where they were maintained at 4°C until launch.

5.1.2 Operations Timeline

AES-1 launched on Orbital CRS-1 on January 9, 2014 with the samples at 4°C. After being berthed to ISS, about three days later the samples were transferred from the transport CGBA to another CGBA onboard Station waiting at 4°C. Station CGBA was commanded to 30°C and 23 hours later, the first activation took place (introduction of the inoculum into the growth medium) – point A in Figure 5. Nineteen hours later the second activation was conducted (introduction of the antibiotic solution into the growth chamber).

Thirty hours later the experiment was terminated (fixative allowed to mix with the culture). The GAPs containing FPAs with PFA as fixative were stored in Station CGBA, which was then commanded to 20°C. The ones with RNA Later II were placed in the MELFI freezer at -75°C. All of these operations were repeated on Earth for the matched ground controls with the same timing, but 8 hour delayed.

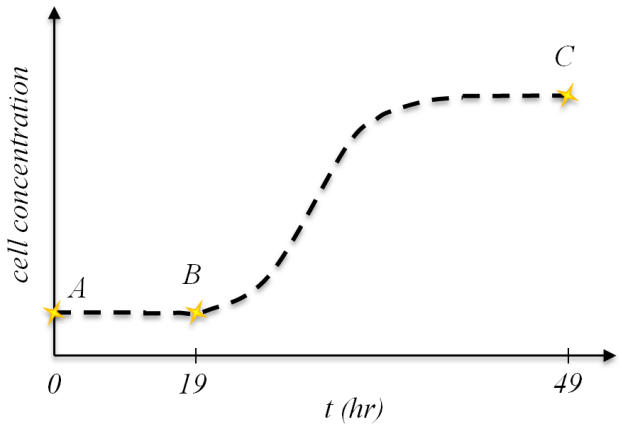


Figure 5. AES-1 operational timeline. The experiment start was indicated by inoculum introduced into the growth medium (A). Nineteen hours later, at the expect end of lag phase under these conditions, the antibiotic was introduced (B).Finally, samples were fixed (C) for post-flight analysis.

Space samples remained stored until their return to Earth. The PFA-fixed samples came back on SpaceX-3, which landed May 18, 2014, and the RNA Later II samples returned on SpaceX-4 on October 25, 2014. A detailed timeline is presented in Appendix 2 and the flight and ground control temperatures in Appendix 3.

Figure 6. Astronaut Mike Hopkins operating an AES-1 GAP onboard ISS. The cranking motion allowed for the fluid in a chamber to travel to the next within each of the eight FPAs.

5.1.3 Replicates and Ground Controls

Each FPA contained a specific testing condition, i.e. one of the two types of antibiotics at a specific concentration and one of the two fixatives. The flight set consisted of 128 FPAs, sixteen of them without antibiotic and used as flight controls – eight to measure cell concentration at experiment start and the other eight at time of what otherwise would be antibiotic introduction ("A" and "B", respectively, on Figure 5). The latter eight were originally purposed to serve as negative controls for the antibiotic interrogation, i.e. to be antibiotic-free samples that would be fixed at experiment end ("C" on Figure 5). However, it was decided to assess if the cell concentrations were the same in space and ground controls at the time of antibiotic introduction (to make sure this would not be a confounding factor on the antibiotic effectiveness results); the six FPAs that were to be used at time "C" were

actually used on time “B”. The lack of antibiotic-free samples at time “C” was overseen until sample return to Earth.

Each testing condition had four replicates for statistical significance. Ground controls consisted of a similar set of testing conditions summing 168 FPAs. Since the lowest minimum concentrations of antibiotic required to inhibit growth on Earth had already been determined as 25 μg/mL and 1 μg/mL for Gentamicin Sulfate and Colistin Sulfate, respectively, concentrations higher than 75 μg/mL and 4 μg/mL for those drugs were not prepared as part of the ground controls. To replicate the temperature changes that took place on orbit during operations, ground controls were stored and operated inside BioServe’s environmental test chamber, which can mimic the Station CGBA’s humidity and temperature profiles.

5.2 RESULTS

5.2.1 Bacterial Population Density and Lag Phase

Cell concentration in the spaceflight and ground control samples was measured at different time points in the experiment, including at the start (following inoculum), in the acceleration phase (in between lag and exponential phases, when the antibiotic was introduced), and at the test end (stationary phase). A paired t-test was conducted to compare these values in spaceflight and matched ground control samples. No statistically significant difference was observed in cell count at experiment start or acceleration phase in between spaceflight ($M = 6.91 \times 10^6$ cells/mL, $SD = 7.98 \times 10^6$ cells/mL) and matched ground controls ($M = 7.88 \times 10^6$ cells/mL, $SD = 1.65 \times 10^6$ cells/mL) ($t(10) = -0.24$, $p = 0.8151$). This indicates that the spaceflight and matched ground control cultures had similar cell concentrations at the time of antibiotic introduction. Although these results could suggest

that there were no changes in cell lag phase duration, this is actually not definitive, as this data is from one point in time and to determine the effective end of lag phase data from several time points would be needed.

5.2.2 Launch Delays and their Impact on the Colistin Sulfate Samples

The launch of the Orbital CRS-1 mission, carrying AES-1, was delayed from December 20, 2013 to January 9, 2014 due to a failure on an ammonia pump on ISS, extreme cold weather at the launch site, and high radiation space environment due to solar activity. During these delays, all the samples remained at 4°C, which is the recommended storage temperature for Gentamicin Sulfate but not for Colistin Sulfate, which needs to be maintained at -20°C when diluted to the AES-1 concentrations (1-7 μg/mL). This unplanned and extended storage at 4°C likely degraded the quality of the Colistin Sulfate solutions, as no trends were observed as a function of drug concentration on either the ground or spaceflight cultures (Figure 7). Nevertheless, differences were observed between spaceflight samples with respect to their matched ground controls. In other words, although the "drug concentration" independent variable could not be assessed with the Colistin Sulfate cultures, the "gravitational environment" independent variable could.

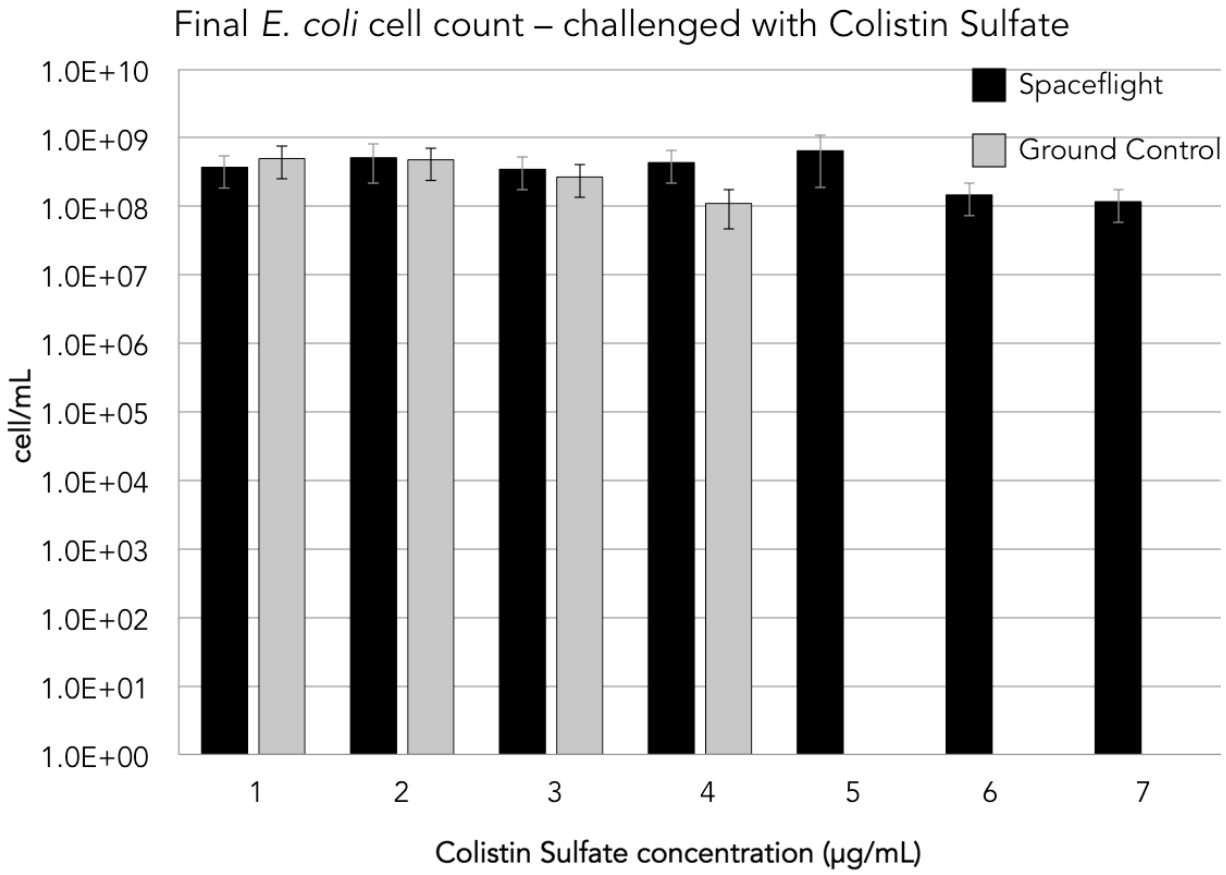


Figure 7. Final cell count when challenged with Colistin Sulfate. No trends in final cell count were observed as a function of drug concentration. It is believed that the long-term storage at 4°C – due to a series of launch delays – degraded the quality of the drug solutions.

5.2.3 Bacterial Growth When Challenged With Gentamicin Sulfate

Seven concentrations of Gentamicin Sulfate (25 to 175 µ/ml) were tested in space but only the three lowest concentrations were evaluated in ground controls. The four higher concentrations were deemed unnecessary as it had already been determined that the lowest drug concentration (25 µg/ml) was sufficient to inhibit bacterial growth under the test conditions. Spaceflight cell concentrations at the end of the experiment were higher than their respective ground controls in all cases. A paired t-test showed that, when challenged with 25 µg/ml of Gentamicin Sulfate, there was a seven-fold increase in final cell count on spaceflight with respect to ground ($t(5) = 13.03$, $p < .0001$). This increase was 41-fold ($t(5) =$

19.75, $p < .0001$) and 18-fold ($t(6) = 5.74$, $p = 0.0012$) for the next two antibiotic concentrations (50 and 75 µg/ml), respectively. Conducting a paired t-test on these three lowest concentrations as a single group showed that there was a 13-fold increase in final cell count in space with respect to ground ($t(20) = 6.77$, $p < .0001$). Details can be seen in Figure 8.

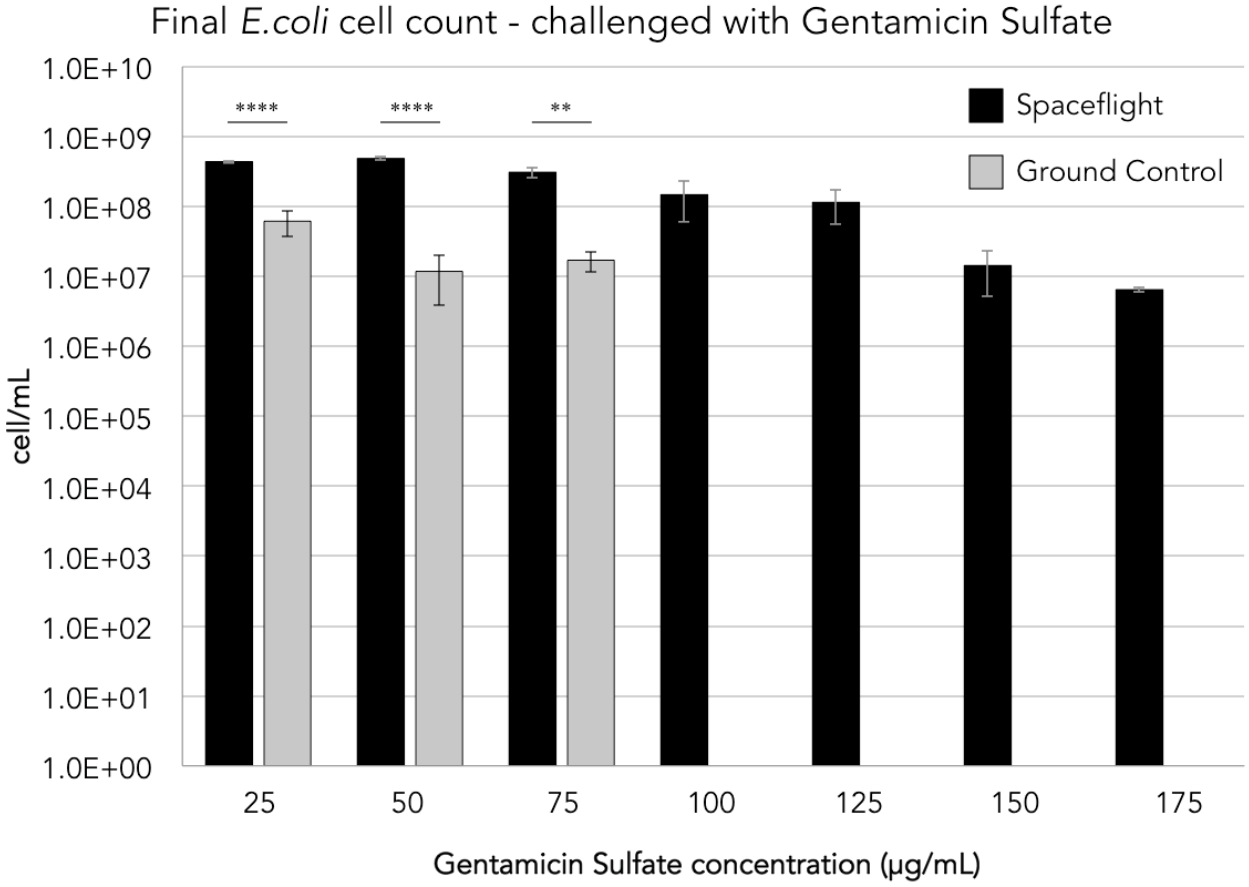


Figure 8. Final cell count when challenged with Gentamicin Sulfate. Higher cell population counts were observed in space with respect to ground (13-fold increase in average). Although there appears to be a decrease in magnitude at 150 and 175 µg/ml with respect to 125 µg/ml and less, this is misleading as accurate values were hard to acquire either by cell count or optical density due to cell aggregation in these samples. It is estimated that values at 150 and 175 µg/ml were roughly equivalent to that of 125 µg/ml. Bars indicate standard error, $n = 4$ for all except for spaceflight at 25 and 50 µg/ml ($n = 3$, each), and 175 µg/ml ($n = 2$), as only the samples for which it was certain that the antibiotic was fully introduced were considered.

5.2.4 Bacterial Culture Morphology

From initial assessment of the samples upon their return to Earth, pronounced cell aggregation was the most prominent phenomenon observed. Spaceflight samples with Gentamicin Sulfate concentrations of 125 µg/ml or higher exhibited cell aggregation to the point that the culture essentially became a contiguous, single cluster, as seen in Figure 9C. This behavior was not observed on the ground controls. Similarly, spaceflight samples challenged with the three highest Colistin Sulfate intended concentrations – 5, 6 and 7 µg/ml – showed cohesive growth, again, not seen in the matched ground control samples. This is in contrast to the usually uniform fine turbidity cultures observed at 1g.

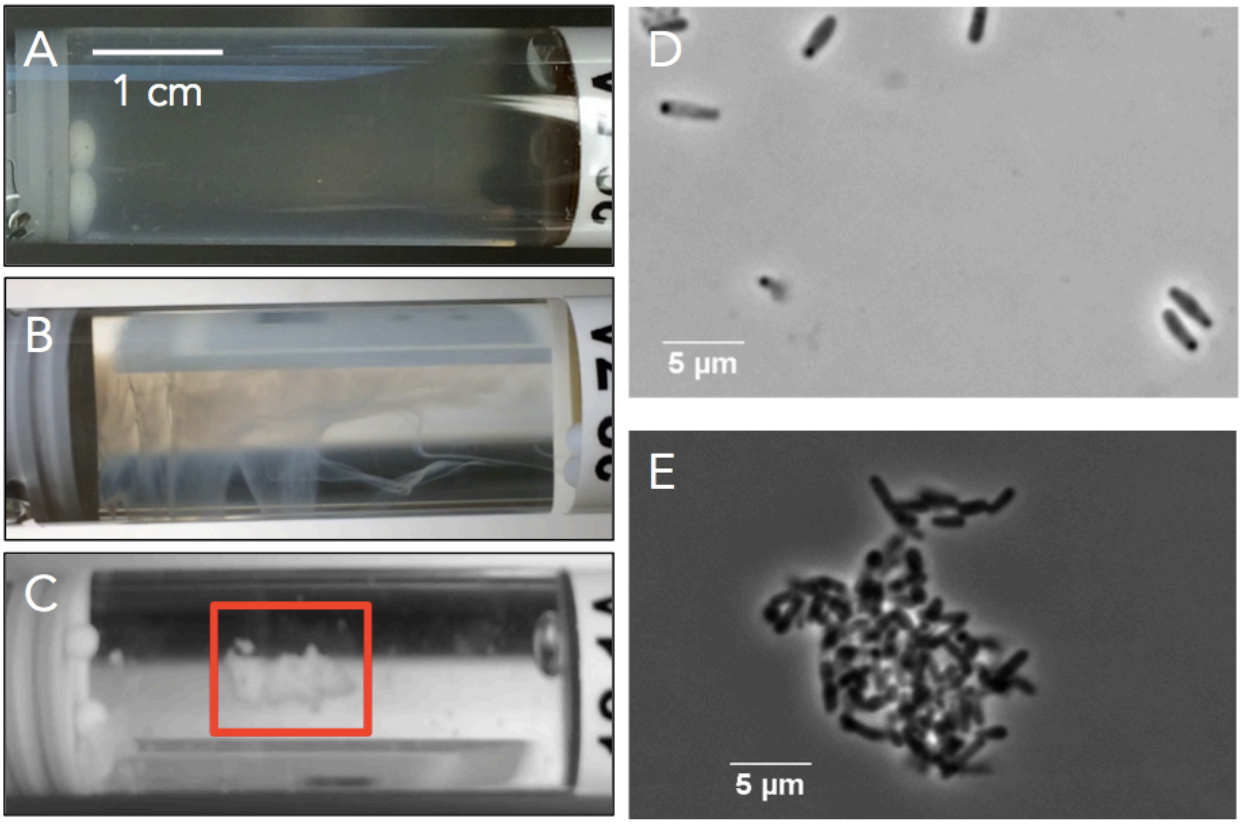


Figure 9. Liquid Culture Morphology. *E. coli* commonly grows in ME growth medium with uniform fine turbidity as seen in the ground control (A). Some of the spaceflight samples challenged with Colistin Sulfate showed cohesive, viscous clouds of cells (B) not observed on the matched ground controls. Similarly, spaceflight samples challenged with the highest

concentrations of Gentamicin Sulfate tended to aggregate into a single cluster (box in (C)). The images on the right show *E. coli* cultures under phase contrast microscopy. Image (D) shows regular growth on a ground control sample challenged with the lowest concentration of Gentamicin Sulfate (25 μg/ml) and (E) a spaceflight sample challenged with the highest concentration of the same drug 125 μg/ml. The differences in cell size and aggregation are noticeable. Images were taken with a Nikon E600 Widefield Microscope.

5.2.5 Cell Size

One-way ANOVA were conducted to assess the role of drug concentration on a) cell length and b) diameter for the four different combinations of drug type (Gentamicin Sulfate and Colistin Sulfate) and gravity environment (spaceflight and ground control). There were no statistically significant differences in either condition, suggesting that cell size is independent of drug concentration – the worst case scenario being [$F(6,3) = 2.709$, $p = 0.2220$] and the best [$F(6,5) = 0.202$, $p = 0.962$]. However, both cell length and diameter differed based on the gravity environment. Based on these two observations, data sets were pooled together in two groups: spaceflight and ground controls (as seen in Figure 10) and paired t-tests were conducted to compare their role in cell length and cell diameter. There was a significant difference in cell length in spaceflight (M = 1.660 μm, SD = 0.288 μm) and ground controls (M = 2.353 μm, SD = 0.603); ($t(310) = -2.07$, $p = 0.0389$). Similarly, there was a significant difference in cell diameter in spaceflight (M = 0.627 μm, SD = 0.084 μm) and ground controls (M = 0.809 μm, SD = 0.131); ($t(310) = -2.33$, $p = 0.0203$). In other words, there were reductions in cell length and diameter to 71% and 78% of their sizes on Earth, respectively. The decrease in cell length and diameter translated into an average reduction of cell surface area to 54% of its value on Earth ($t(310) = -2.53$, $p = 0.0119$); maximum cross section area was also reduced to 54% of that observed on the ground ($t(310) = -2.48$, $p = 0.0139$); and cell volume was reduced to a 41% of the ground controls value ($t(310) = -2.35$, $p = 0.0196$), as seen in Figure 11.

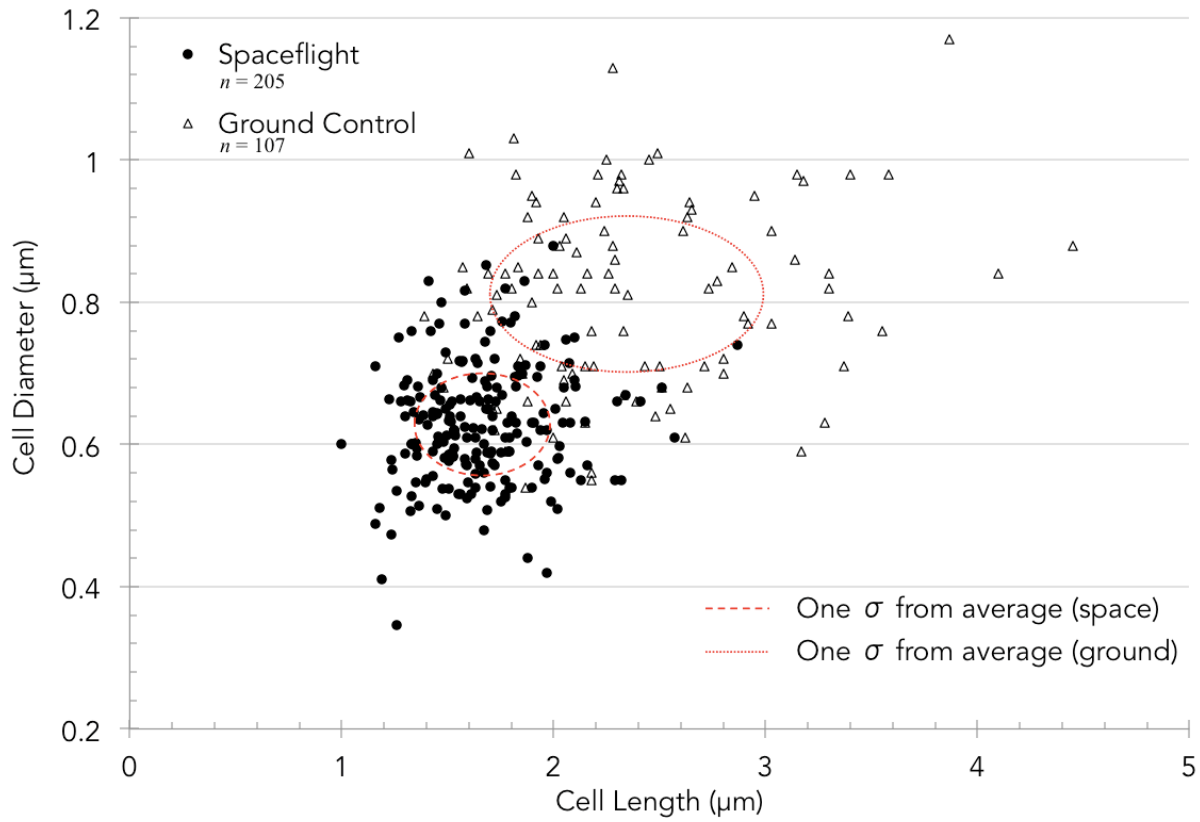


Figure 10. Cell length vs. cell diameter. In space, cells grew to be only a 71% of the length of the ground controls ($t(310) = -2.07$, $p = 0.0389$) and 78% of their diameter ($t(310) = -2.33$, $p = 0.0203$).

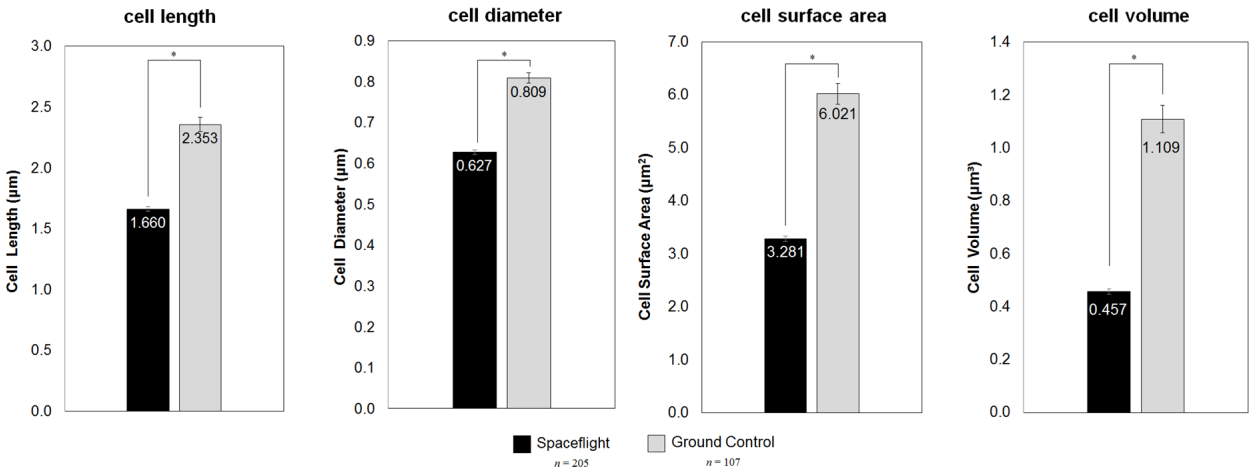


Figure 11. Cell size differences in between spaceflight and matched ground controls. The decrease in cell length and diameter translated into an average reduction of cell surface area to 54% of its value on Earth ($t(310) = -2.53$, $p = 0.0119$). Maximum cross section area was also reduced to 54% of that observed on the ground ($t(310) = -2.48$, $p = 0.0139$) and cell volume to 41% of the ground control value ($t(310) = -2.35$, $p = 0.0196$).

5.2.6 Cellular Envelope Thickness

Transport Electron Microscopy (TEM) images of AES-1 samples (as seen in Figure 12) are being taken at the University of Copenhagen to measure and analyze cell envelope thickness changes. However, at the time of the defense of this thesis, not enough data had been produced to make any conclusions (Figure 13). The preliminary data that is available at this time suggests that there was an increase in cellular envelope thickness in space – what is not clear yet, however, if this was due to the gravity environment or because of antibiotic concentration. When all the data is produced, one-way and two-way ANOVA will be conducted to assess cell envelope thickness changes as a function of drug type, drug concentration and gravitational environment. The TEM images will also be used to investigate if other visual changes in the cell envelope are present.

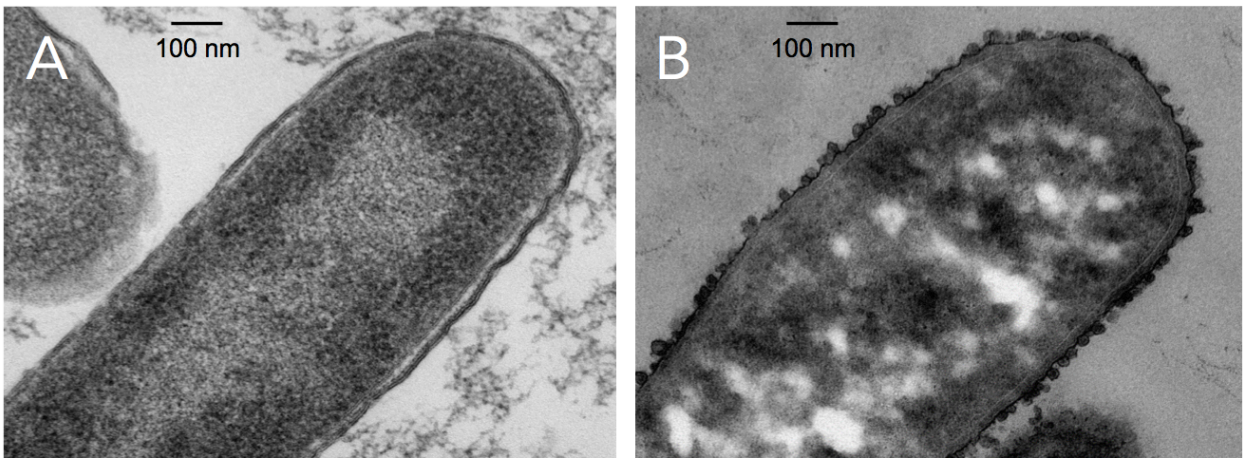


Figure 12. Transmission Electron Microscopy (TEM) images of *E. coli*. *E. coli* challenged with 50 µg/mL of Gentamicin Sulfate on Earth (A) and another challenged with 175 µg/mL of Gentamicin Sulfate in space (B). It cannot yet be elucidated if the differences observed are due to the gravitational environment or because of drug concentration, but the fact that there are differences is observable. Images taken with a Philips CM 100 TEM microscope operated at an accelerating voltage of 80 kV and equipped with an OSIS Veleta digital slow scan 2k x 2k CCD camera.

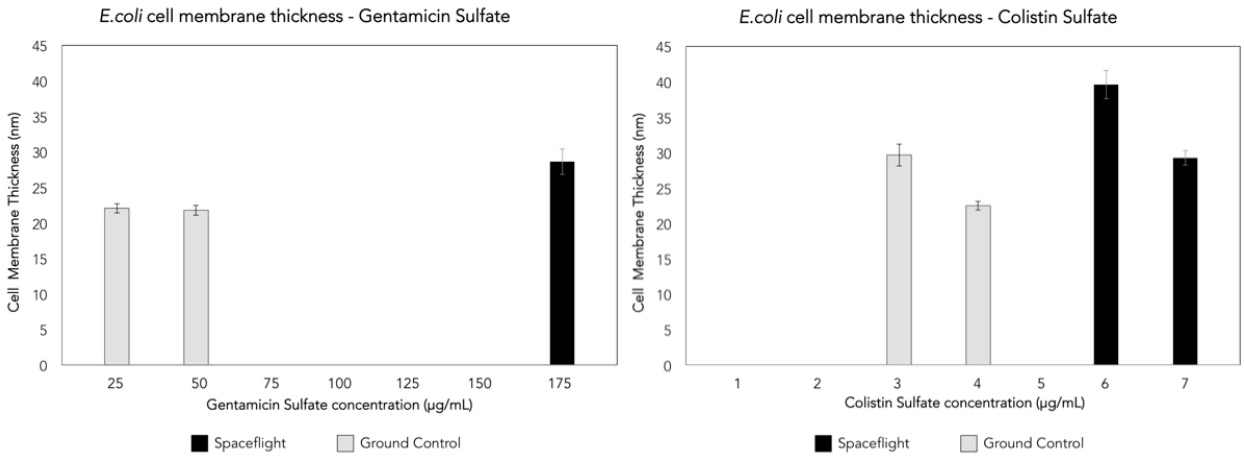


Figure 13. Preliminary cell envelope thickness data. No appropriate statistical analysis was possible at the time of publishing this thesis. However, data for the missing testing conditions was being acquired at time of print.

5.3 CONCLUSIONS

No statistically significant difference was observed in cell population count at experiment start or acceleration phase (n = 12), which suggests that cell concentration was similar at the time of antibiotic introduction in the space and ground cultures. Although this could also suggest that no changes occurred in lag phase duration, data from only one point in time is insufficient to make such claim, as it is not determined when the acceleration phase actually started. Spaceflight cell concentrations at the end of experiment were always higher than their respective ground controls (13-fold increase in average), as has been typically observed. Spaceflight samples with Gentamicin Sulfate concentrations of 125 µg/ml or higher exhibited cell aggregation to the point that the culture essentially became a contiguous, single cluster. This behavior was not observed for the ground controls. This phenomenon may be related to that of enhanced biofilm formation observed in space, as described by Kim et al. (2013), and that reportedly can increase bacterial resistance to antibiotics (Hall-Stoodley, Costerton, & Stoodley, 2004). There was an average reduction of cell surface and cross section areas to 54% of their values on Earth, which in turn, can

proportionally decrease the rate of antibiotic molecules reaching the bacterial cell surface. The average spaceflight cell volume was also reduced to 41% its 1g value. On the other hand, this spaceflight decrease in size translated into a 34% increase in cell diffusion rate with respect to 1g. Work has been published on mathematical and computational models describing the role of these gravity-driven phenomena around a cell (Benoit & Klaus, 2005; Klaus et al., 2004, 1997), however, these models assumed that the cell size was the same on Earth as in space. This phenomenon may be related, in part, to why a higher concentration of antibiotics was needed in space to inhibit bacterial growth, as the antibiotic molecules – driven mainly by Brownian motion (Klaus, 1994) – would have a smaller target.

5.4 DISCUSSION

The samples were fixed at completion of the experiments to avoid bacterial re-adaption to gravity. Post-flight analysis showed no statistically significant difference in cell count at experiment start or acceleration phase, suggesting that the ratio of antibiotic molecules per bacterium remained similar between flight and ground controls.

Although minimal, some growth was observed on ground samples with Gentamicin Sulfate concentrations of 25 μg/ml and above. This was likely due to a slight decrease in drug efficacy since there was approximately one month between antibiotic solution preparation and experiment start due to a series of unexpected launch delays. This also caused the final cell count data produced from the samples challenged with Colistin Sulfate to be inconclusive as the effective drug concentration at time of experiment start was unknown and no statistically significant difference or trend were observed. It is believed that this is because at these low concentrations, this drug is unstable at the pre-launch storage temperature of 4°C. Potential changes to drug efficacy due to being in the space environment are disregarded, as the experiment was in space only for two days prior to

experiment start and chemical changes of pharmaceuticals in spaceflight are believed to be a longer term phenomenon, as explained in (Du et al., 2011).

Cellular aggregation was observed on the spaceflight cultures challenged with the highest concentrations of Gentamicin Sulfate, and cohesive growth was noted on the space samples challenged with the highest intended Colistin Sulfate concentrations. The formation of clumps through cellular aggregation have been reported on *Salmonella typhimurium* flown to space, together with expression changes on the *wca/wza, ompA* and *fimH* genes, which are associated with cell surface alterations related to biofilm formation (Wilson et al., 2007). Clumping behavior was also previously observed to occur in *E. coli* samples used in early space shuttle experiments conducted by BioServe (unpublished data). This phenomenon may be related to that of enhanced biofilm formation observed in space, as described by Kim et al. (2013), and that reportedly can increase bacterial resistance to antibiotics (Hall-Stoodley et al., 2004). It is hypothesized that this aggregation may have permitted the cells on the surface of the cluster to protect those in the core from antibiotic exposure. However, this would have also hindered glucose from reaching the latter. Nevertheless, cell aggregation at high antibiotic concentrations suggests that there may be changes occurring to physical properties of the cellular envelope and should be further investigated.

The decrease in cell length and diameter translated into an average reduction of cell surface area to 54% of its value on Earth ($t(310) = -2.53$, $p = 0.0119$). Maximum cross section area was also reduced to 54% of that observed on the ground ($t(310) = -2.48$, $p = 0.0139$). Smaller cross section and cell surface areas proportionally reduce the rate of antibiotic molecules reaching a bacterial cell.

In addition, in microgravity, Brownian motion governs the rate of contact, whereas on Earth, gravity-driven phenomena – namely buoyancy and sedimentation – contribute to

cell movement, as explained in Klaus (2004). Work has been published on mathematical and computational models describing the role of these gravity-driven phenomena around a cell (Benoit, 2005; Klaus et al., 2004, 1997), however, these models assumed that the cell size was the same on Earth as in space.

CHAPTER 6

GENE EXPRESSION CHANGES IN *E. COLI* CHALLENGED WITH GENTAMICIN SULFATE IN SPACEFLIGHT

Gene expression observed on the spaceflight samples with respect to their matched ground controls was analyzed in two distinct ways. One of them was the per-scenario approach. Spaceflight samples challenged with a specific type and concentration of drug were compared to the ground controls that were cultured under the same conditions. There are three scenarios for the cases where *E. coli* was challenged with Gentamicin Sulfate: 25, 50 and 75 μg/mL, each one having their own set of up- and down-regulated genes. Because the number of genes that were under or overexpressed is in the order of magnitude of the thousands, the genes that were regulated by at least 10x were listed. The complete lists of differentially expressed genes can be found in Appendix 5. The other approach for this analysis is in a per-drug basis, where a single set of up- and down-regulated genes is developed from the three spaceflight samples groups (25, 50 and 75 μg/mL). This set is not the addition of the three individual sets, but a list of the overlapping genes over or underexpressed among *all* three groups.

As seen in Figure 14, out of the 4,320 genes in this strain of *E. coli*, the spaceflight samples challenged with 25 μg/mL showed no expression changes on 57% (2483) of the genes, while this value was 49% and 91% for the sets with 50 μg/mL and 75 μg/mL,

respectively. Gene expression changes tended to down-regulation on the 25 µg/mL samples, while it did to up-regulation on the two other sets.

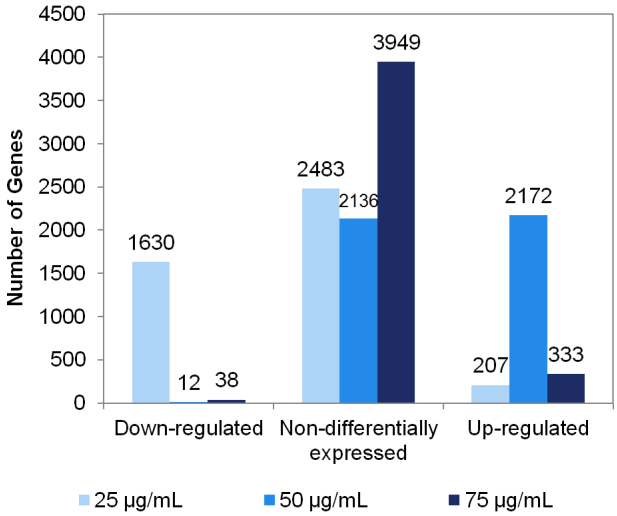


Figure 14. Number of genes down- and up-regulated in the spaceflight samples with respect to matched ground controls.

6.1 METHODOLOGY

At the end of the experiment, about half of the AES-1 samples were fixed in RNA Later II (Life Technologies, Cat No. B7024, Carlsbad, CA, USA) at a 0.6 fixative/sample v/v ratio. After their return to Earth on SpaceX-4 (October 25, 2014), the samples were handed over to HudsonAlpha (HA) for transcriptomic and genomic analyses. Gene expression was assessed by HA via RNAseq performed on the Illumina HiSeq platform. Three of the four replicates were analyzed and expression means were produced. Significant expression was considered as anything larger than a $|2|$-fold change with respect to its respective matched ground control. HA provided me with lists of genes that were differentially expressed in a per-scenario approach (of the 25 µg/mL, 50 µg/mL, and 75 µg/mL Gentamicin Sulfate test scenarios), as described above, for me to analyze and to elucidate the correlations between the phenotypic and gene expression observations. Gene functions, as well as their related molecular functions, biological processes, cellular components, predicted protein classes,

and pathways were identified using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System from the Gene Ontology Reference Genome Project (GORGP, 2015). At the time of defending this dissertation, data from the samples challenged with Gentamicin Sulfate concentrations higher than 75 µg/mL or with Colistin Sulfate were not yet available and therefore this analysis is limited to the samples challenged with Gentamicin Sulfate.

6.2 PER-DRUG APPROACH

The 25 µg/mL samples showed an up-regulation on 207 genes and a down-regulation on 1630. These values were 2172 and 12 for the 50 µg/mL set; and 333 and 38 on the 75 µg/mL samples (up-regulation and down-regulation, respectively). This translates to 2281 overexpressed and 1664 underexpressed individual genes throughout the all sets. An initial step to analyze such a large database was to find the genes that were *commonly* (overlapping) over or underexpressed. As seen in Figure 15, 28 genes were up-regulated in the three groups, and only one gene was down-regulated throughout the three sets.

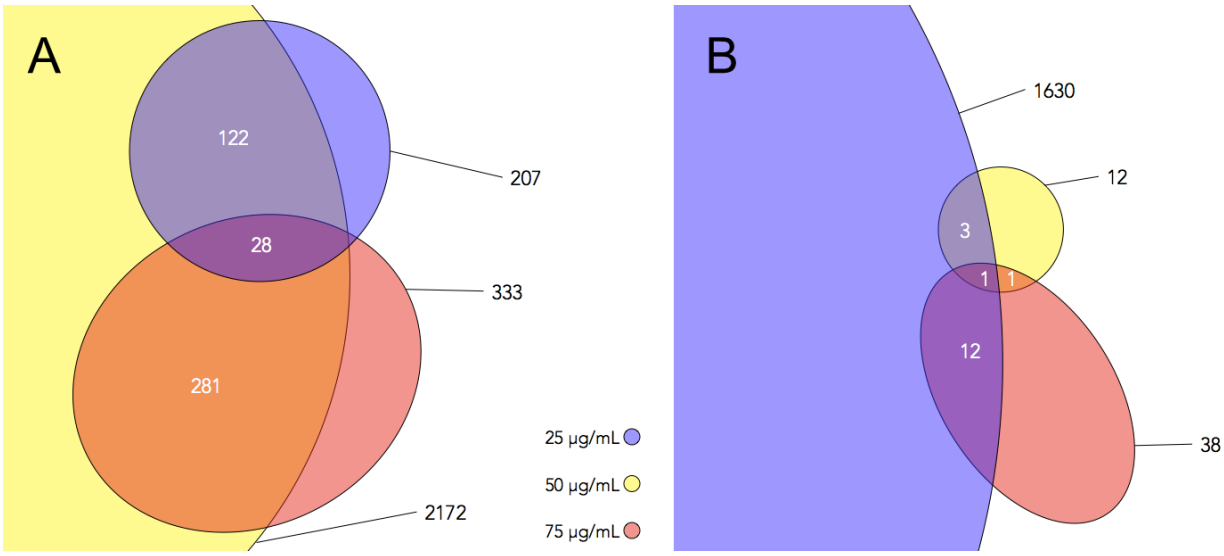


Figure 15. (A) shows that, from the 2172 overexpressed genes in the 50 µg/mL set (yellow bubble), 122 were also up-regulated in the 25 µg/mL group, 281 were in common with the

75 μg/mL set, and only 28 were overexpressed in all three groups. A similar analysis is shown in (B); only 1 gene was commonly down-regulated among all three groups.

Table 4 lists the 28 genes that were commonly up-regulated. This list includes four *opp* genes (B,C,D, and F), which synthesize proteins related with oligopeptide transport into the cell; *E. coli* utilizes oligopeptides as a source of carbon and energy (Andrews & Short, 1985). It has been reported that a reduction in *oppA* expression confers *E. coli* with resistance to several aminoglycosides (Gentamicin Sulfate is an aminoglycoside), as it behaves as a carrier for the antibiotic (Acosta, Ferreira, Padilla, Ferreira, & Costa, 2000). Interestingly, *oppA* was *over*expressed in the 50 μg/mL (5.87x), 75 μg/mL (2.81x) sets, and not differentially expressed in the 25 μg/mL group. Table 4 also lists the *sucABCD* genes as being upregulated in the spaceflight samples in between 2.05x and 4.39x. These genes are related with processes involving succinyl-Co-A, a combination of succinic acid and coenzyme A. It has been reported that Suc^- phenotypes of *E. coli* have cross-resistance to aminoglycosides (Collis & Grigg, 1989). Again, in this case, these genes were *over*expressed. Five *thi* genes (E, F, G, H, and S) were not only up-regulated but were also the ones that were increased the most from the list of 28 (in between 24.88x and 32.41x). These genes are associated with catalytic activity, and several metabolic processes, but no correlation has yet been found with the observed phenotypic changes on the spaceflight samples. The *malE* gene plays a role in the transport of maltose and other substrates across cellular membranes and is further discussed in context in section 6.3.2.

Table 4. List of overlapping up-regulated genes throughout all three groups, their respective functions and the value of its overexpression in space with respect to matched ground controls.

Gene name	Function	25 µg/mL	50 µg/mL	75 µg/mL
malE	Maltose-binding periplasmic protein	2.81	43.8	24.09
thiS	Sulfur carrier protein	3	32.41	3.97
thiG	Thiazole synthase	2.34	30.48	5.32
thiF	Sulfur carrier protein ThiS adenylyltransferase	3	28.87	4.93
thiE	Thiamine-phosphate synthase	2.84	28.59	4.29
thiH	2-iminoacetate synthase	2.37	24.88	5.06
yiaG	Uncharacterized HTH-type transcriptional regulator	11.34	8.45	2.94
yeaQ	UPF0410 protein	7.04	6.26	2.83
bfr	Bacterioferritin	3.8	8.19	2.93
ykgC	Probable pyridine nucleotide-disulfide oxidoreductase	2.55	8.13	4.02
yjbE	Uncharacterized protein	2.95	5.62	4.23
oppF	Oligopeptide transport ATP-binding protein	4.08	4.77	2.73
oppD	Oligopeptide transport ATP-binding protein	4.36	4.27	2.6
adhE	Aldehyde-alcohol dehydrogenase	2.61	5.73	2.61
sucD	Succinyl-CoA ligase [ADP-forming] subunit alpha	4.22	4.39	2.29
yeaG	Uncharacterized protein	3.44	4.73	2.69
gltA	Citrate synthase	2.35	4.64	3.38
ydiZ	Uncharacterized protein	2.42	4.58	3.11
yehE	Uncharacterized protein	3.06	3.25	3.79
oppB	Oligopeptide transport system permease protein	2.67	4.68	2.63
oppC	Oligopeptide transport system permease protein	3	4.3	2.52
sucC	Succinyl-CoA ligase [ADP-forming] subunit	3.33	4.16	2.22
sucB	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	3.91	3.6	2.05
yciF	Protein YciF	2.91	3.37	3
fimZ	Fimbriae Z protein	2.53	4.38	2.07
sucA	2-oxoglutarate dehydrogenase E1 component	2.7	4.07	2.11
agp	Glucose-1-phosphatase	2.92	3.42	2.17
tauB	Taurine import ATP-binding protein	2.06	2.43	2.45

Only one gene, *cusF*, was found to be commonly under-expressed throughout all the three test sets, as seen in Table 5. This gene synthesizes the *cusF* protein, which is related with a cation efflux system. Although no correlations have been found so far, more in-depth research is needed to elucidate if there is a correlation between this process and the phenotypic observations made on the spaceflight samples.

Table 5. List of overlapping down-regulated genes throughout all three groups, their respective functions and the value of its overexpression in space with respect to matched ground controls.

Gene name	Function	Fold		
		25 µg/mL	50 µg/mL	75 µg/mL
cusF_1	Cation efflux system protein CusF	-2.13	-3.75	-2.76

The 29 differentially expressed genes were analzyed as a group using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System. The protein classes synthesized by 20 of the 29 genes were identified and about half of them were related with transporters and transferases (see Figure 16). Eleven out of the fifteen identified molecular functions were associated with catalytic activity in general, while three of them were with transporter activity. More than half (11 out of 20) of the biological processes impacted by the differential gene expression were correlated with metabolism. Only two cellular component groups were identified as affected: “cell part”, and “membrane”, i.e. no changes were predicted on macromolecular complexes, extracellular region, or organelles, for example. The pathway impacted the most from this set of genes was the TCA (cytrate) cycle.

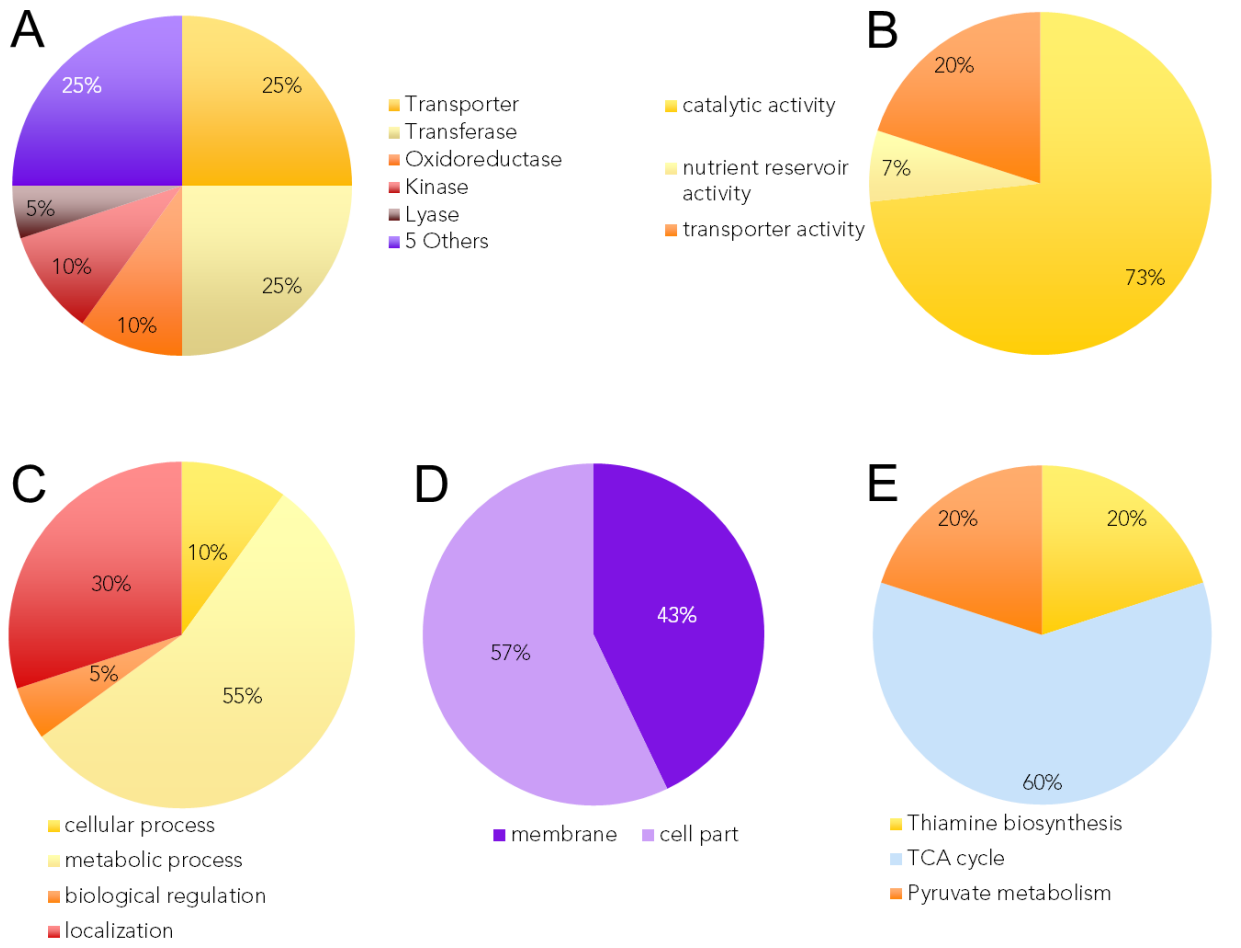


Figure 16. An analysis of the 29 commonly differentiated genes throughout the test groups allow to see the distribution of the proteins they synthesize (A), their molecular functions (B), biological processes (C), cellular components (D), and related pathways (E).

6.3 PER-SCENARIO APPROACH

6.3.1 Twenty-five µg/mL

The samples challenged with the lowest concentration of Gentamicin Sulfate (25 µg/mL) showed a down-regulation of 1630 genes (38% of the total), the up-regulation of 207 genes (5%), while 2483 were non-differentially expressed (57%). From the 207 overexpressed genes, ten did so in an increase larger than 10x (see Table 6). Although several of them are still uncharacterized, the function of most of them has been identified;

for example, *gadE* (20.62x) is associated with acid resistance and is analyzed in more detail in section 6.7. The *wrbA* gene (22.71x) synthesizes the WrbA flavoprotein, which is believed to have a role in oxidative stress defense and/or cell signaling (Kishko et al., 2012) and is also acid-induced (Tucker, Tucker, & Conway, 2002). A more throughout analysis of this gene, and the potential role of oxidative stress, is presented in section 6.6; it is also discussed in section 6.7. Similarly, the overexpression of gene *yccJ*, another acid-induced gene, is analyzed in section 6.7. The *ecnA* gene (-34.31x), the antidote to the *ecnB* toxin gene (15.7x), was the single most underexpressed gene in this set. *ecnAB* is an antidote/toxin gene pair (also known as addiction molecules) that control apoptosis, or programmed cell death during starvation conditions.

Table 6. List of genes overexpressed at least by a 10-fold in the 25 μg/mL samples.

Gene name	Function	Fold
yccJ	Uncharacterized protein	26.63
wrbA	Flavoprotein	22.71
gadE	Transcriptional regulator	20.62
yegP	UPF0339 protein	17.92
yhcO	Uncharacterized protein	17.25
ecnB	Entericidin B	15.7
hyaC	Probable Ni/Fe-hydrogenase 1 B-type cytochrome subunit	13.65
hlyE	Hemolysin E, chromosomal	13.12
hyaD	Hydrogenase 1 maturation protease	12.2
yiaG	Uncharacterized HTH-type transcriptional regulator	11.34

From the 1630 underexpressed genes, 41 did so in a decrease smaller than -10x. Such a long list defeats the purpose of this synthesis, but the complete list of underexpressed genes can be found in Appendix 5. In spite of the large number (1837) of differentially expressed genes, they were assessed as a single group to characterize their role on the bacterial processes, as seen in Figure 17. It is noteworthy that 538 out of the 949 (34%) genes identified to affect molecular functions were associated with catalytic activity; and 661 out of 980 (67%) of those determined to impact biological processes were with

metabolic processes. Five different cellular components are predicted to be affected by the 205 genes that have been previously characterized: membrane (74), macromolecular complex (9), extracellular region (4), organelle (1), and cell parts (117).

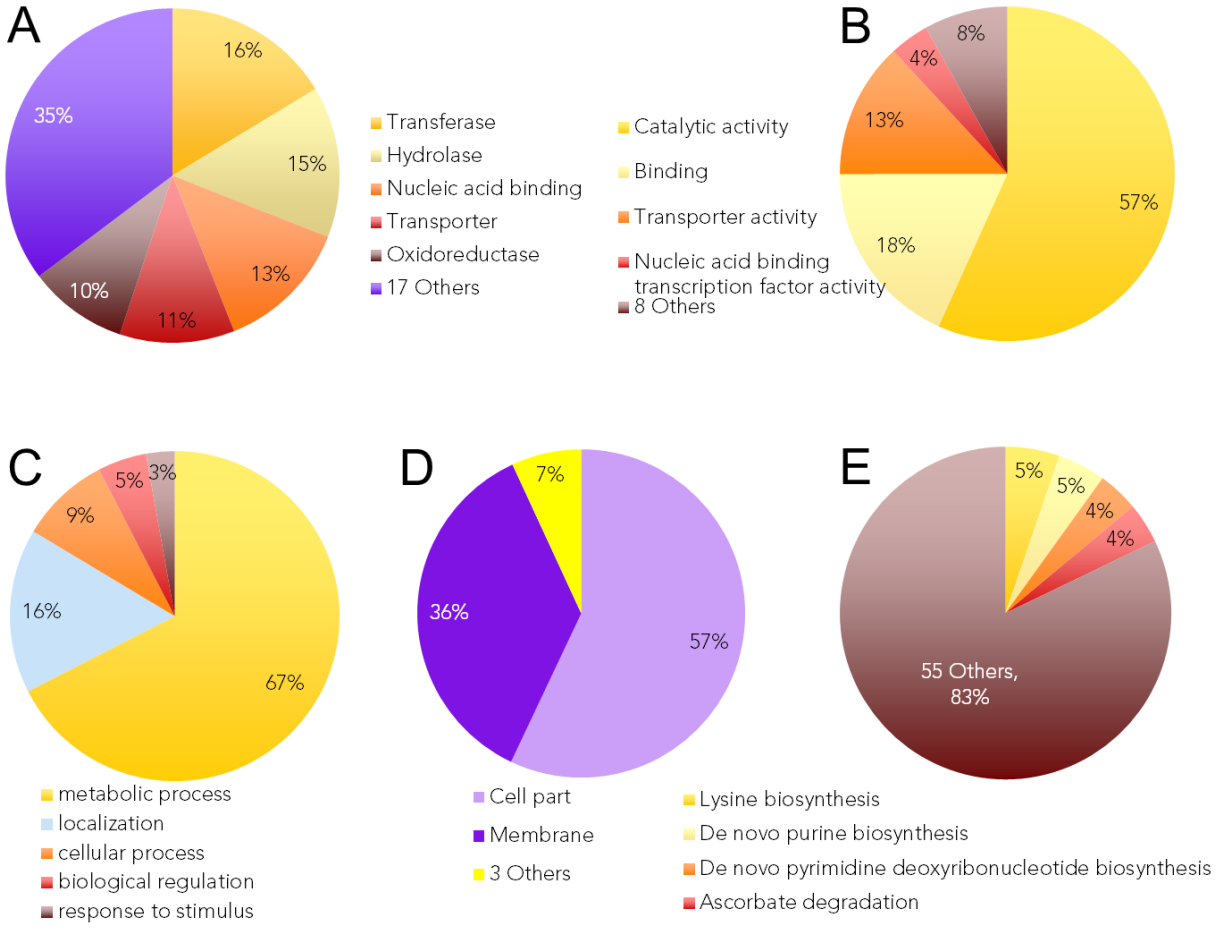


Figure 17. Distribution of the proteins synthesized (A) by all the differentially expressed genes in the 25 µg/mL group, their molecular functions (B), biological processes (C), cellular components (D), and related pathways (E).

6.3.2 Fifty µg/mL

The samples challenged with 50 µg/mL of Gentamicin Sulfate showed down-regulation on only 12 genes (0.3% of the total), up-regulation on 2172 genes (50%), while 2136 were non-differentially expressed. From the 2172 overexpressed genes, 35 did so in an increase larger than 10x (see Table 7). From these 35, the increase in the structural genes

of the *trp* operon *(A, B, C, D,* and *E*) is noticeable because it is five related genes, and because they are the five that incremented in expression the most (in between 45.61x and 69.11x). Through tryptophan synthesis, the biological process associated to *trpA* is cellular amino acid biosynthesis and the subsequent formation of amino acids and organic acids; *trpB* catalyzes the formation of tryptophan from indole and serine; *trpD* synthesizes a glycosyltransferase, an enzyme that catalyzes the transfer of sugar from a donor (sugar nucleotide derivative) to an amino acid acceptor (GORGP, 2015). In general, the *trp* operon is regulated by the presence of tryptophan in the environment through a negative feedback loop (Bertrand, Squires, & Yanofsky, 1976). This suggests that there was a decrease in environmental tryptophan for the *trp* operon to be so overexpressed (up to 69.11x).

Another group of genes that was overexpressed in the 50 µg/mL samples were the *gadABC* and *gadE* genes, which, as mentioned before, are discussed in section 6.7. Additionally, an up-regulation of the *hdeA*, *B* and *E* genes was observed, which is analyzed in section 6.7. The *malE* and *malK* genes were up-regulated 43.8x and 22.39x, respectively. The *malE* gene codes the MalE protein, which transports maltose through inner cell membrane; however, it is essential for the transport of all substrates of the system (Ferenci, 1980). The *malK* gene synthesizes an oligopeptide permease protein, which, similarly to *malE* and the *opp* genes, transports substrates across cellular membranes (GORGP, 2015). The 50 µg/mL samples also showed an up-regulation of several *thi* genes (C, E, F, G, H, and S) in between 24.67x and 32.41x. The first five are the structural genes for Thiamine biosynthetic enzymes while the latter is a sulfur donor in that process (van der Horn, Backstrom, Stewart, & Begley, 1993); thiamine is needed for carbohydrate metabolism (Leonardi & Roach, 2004). None of the 12 down-regulated genes was reduced by a factor smaller than -10x.

Table 7. List of genes overexpressed at least by a 10-fold in the 50 µg/mL samples.

Gene name	Function	Fold
trpA	Tryptophan synthase alpha chain	69.11
trpD	Anthranilate synthase component II	60.78
trpB	Tryptophan synthase beta chain	53.43
trpE	Anthranilate synthase component 1	46.74
trpC	Tryptophan biosynthesis protein TrpCF	45.61
malE	Maltose-binding periplasmic protein	43.8
thiS	Sulfur carrier protein ThiS	32.41
thiG	Thiazole synthase	30.48
hdeB	Acid stress chaperone HdeB	29.08
thiF	Sulfur carrier protein ThiS adenylyltransferase	28.87
thiE	Thiamine-phosphate synthase	28.59
hdeA	Acid stress chaperone HdeA	28.09
gadB	Glutamate decarboxylase beta	25.6
thiH	2-iminoacetate synthase	24.88
thiC	Phosphomethylpyrimidine synthase	24.67
lamB	Maltoporin	23.63
gadE	Transcriptional regulator GadE	23.04
malK	Maltose/maltodextrin import ATP-binding protein MalK	22.39
yhjX	Uncharacterized MFS-type transporter YhjX	21.96
yhiD	Uncharacterized protein YhiD	19.57
gadA	Glutamate decarboxylase alpha	17.49
entC_1	Uncharacterized	15.22
entC_2	Uncharacterized	14.94
hdeD	Protein HdeD	14.86
yjgI	Uncharacterized oxidoreductase YjgI	14.85
entS_2	Uncharacterized	14.44
entS_1	Uncharacterized	13.82
flgB	Flagellar basal body rod protein FlgB	13.03
fes_2	Uncharacterized	12.27
yqeI	Uncharacterized protein YqeI	11.39
fepA_2	Uncharacterized	11.29
gadC	Probable glutamate/gamma-aminobutyrate antiporter	11.11
metA	Homoserine O-succinyltransferase	10.5

Analyzing the 2184 differentially expressed genes together permits to clarify which processes were impacted the most (Figure 18). A protein class could be predicted for 1331 of the genes, of which 231 (17%) were transferases and 189 (14%) were oxidoreductases. From the 1115 genes that could be associated to a molecular function, 688 (62%) conducted catalytic activities and 813 out of 1196 (68%) were involved with metabolic processes.

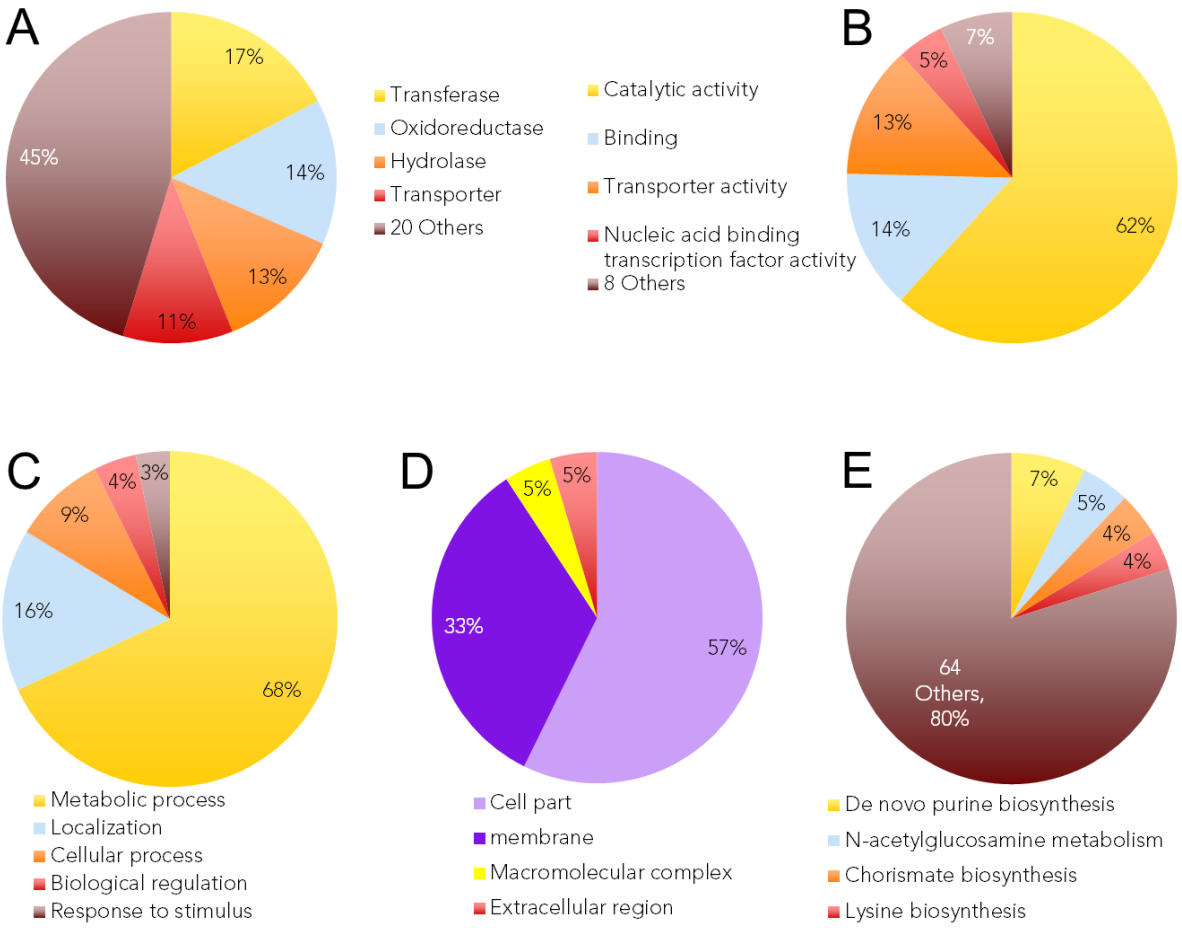


Figure 18. Distribution of the proteins synthesized (A) by all the differentially expressed genes in the 50 µg/mL group, their molecular functions (B), biological processes (C), cellular components (D), and related pathways (E).

6.3.3 Seventy-five µg/mL

The samples challenged with 75 µg/mL of Gentamicin Sulfate showed a down-regulation of 38 genes (1% of the total), the up-regulation of 333 genes (8%), while 3949 were non-differentially expressed (91%). From the 333 overexpressed genes, 12 did so in an increase larger than 10x (see Table 8). The *trp* and *mal* genes have been previously discussed. The "infinity-fold" overexpression of *flxA*, *ybcV*, and *racC* genes is an artifact of the ground controls' expression values being virtually zero; however, the spaceflight values do not elicit a more thorough investigation of these genes. Only one of the 38 down-

regulated genes was reduced by a factor smaller than -10x: *ygeI* (-11.76x), which synthesizes a yet uncharacterized protein (GORGP, 2015).

Table 8. List of genes overexpressed at least by a 10-fold in the 75 μg/mL samples.

Gene name	Function	Fold
flxA	Protein FlxA	∞
racC	Protein RacC	∞
ybcV_2	Uncharacterized	∞
malK	Maltose/maltodextrin import ATP-binding protein MalK	24.28
malE	Maltose-binding periplasmic protein	24.09
lamB	Maltoporin	22.7
trpE	Anthranilate synthase component 1	22.29
trpD	Anthranilate synthase component II	19.45
ykgO	Uncharacterized	14.83
trpC	Tryptophan biosynthesis protein TrpCF	14.06
trpB	Tryptophan synthase beta chain	13.16
trpA	Tryptophan synthase alpha chain	12.46

From the 371 differentially expressed genes in the 75 μg/mL samples, most of the predicted proteins were oxidoreductases (51 out of 245, or 21%) and transferases (17%). Regarding molecular function, almost two thirds (136 out of 208, or 65%) of the characterized genes were associated with catalytic activity, and a similar percentage was with metabolic processes (147 out of 232, or 63%), in terms of their biological function (Figure 19).

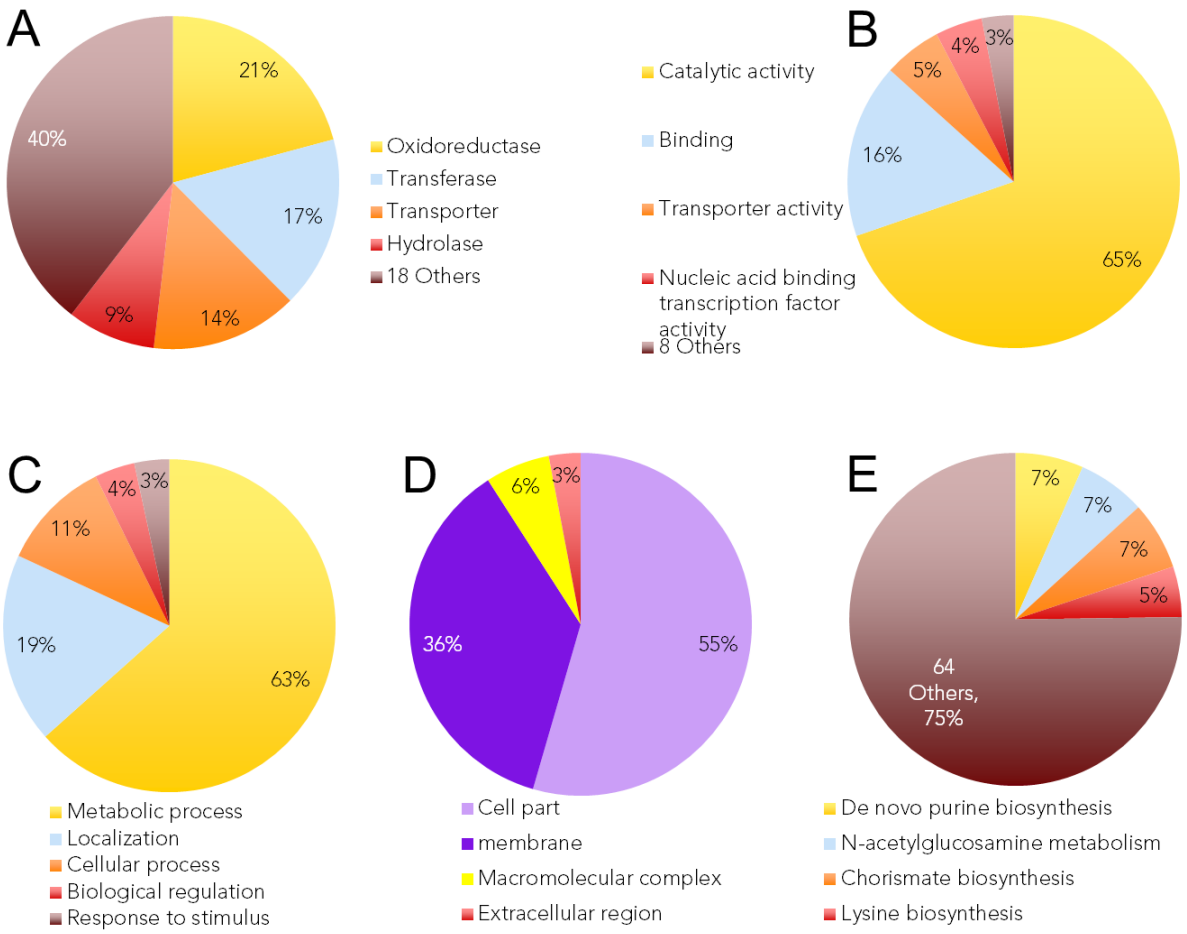


Figure 19. Distribution of the proteins synthesized (A) by all the differentially expressed genes in the 75 µg/mL group, their molecular functions (B), biological processes (C), cellular components (D), and related pathways (E).

6.4 LINEAR ANALYSIS

A linear analysis was conducted to assess the role of Gentamicin Sulfate concentration on different molecular processes. To achieve this, all of the differentially expressed genes (up- and down-regulated) in each of the three sets (25, 50 and 75 µg/mL) were characterized, and then compared against each other in each individual field. Figure 20 shows the predicted impacted proteins from the differentially expressed genes (A) and their molecular functions (B).

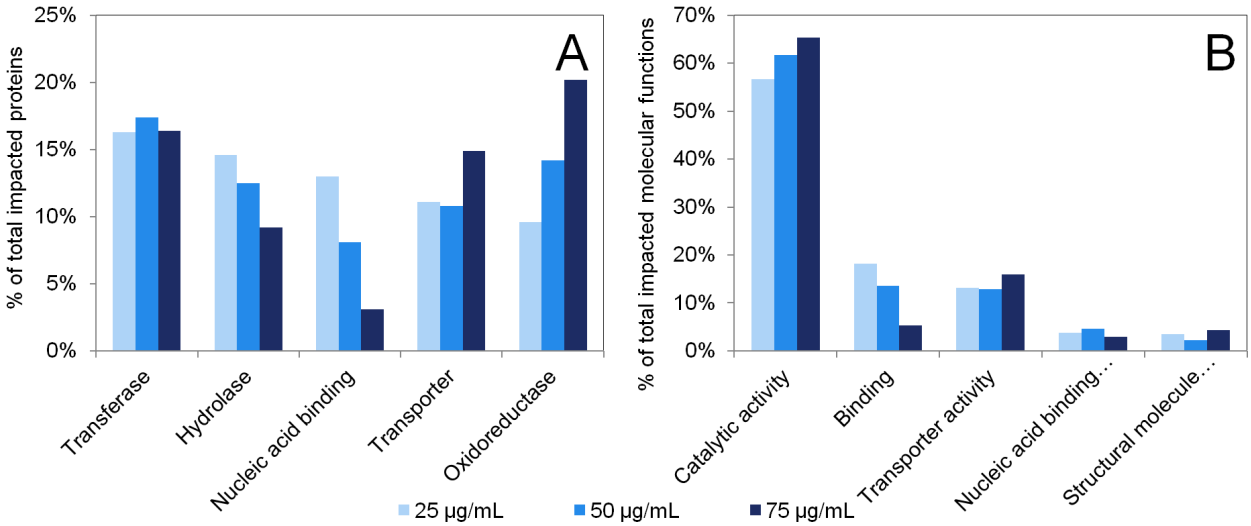


Figure 20. Predicted impacted proteins from the differentially expressed genes (A) and their molecular functions (B). A decrease in synthesis as drug concentration was increased was observed on hydrolases, and nucleic acid binding proteins, while the opposite was true for oxidoreductases. In terms of molecular functions, an increase-trend was observed on catalytic activity, a decrease was noted in binding, and no changes are noticeable in transporter activity, nucleic acid binding transcription factor activity, or structural molecule activity.

As seen in Figure 21, the increase in extracellular region may be related with the cell aggregation observed on the highest concentrations of Gentamicin Sulfate on the spaceflight samples. The investigated differential gene expression had virtually no impact on the cellular organelles, as only one gene out of 205, and one in 237 were associated with them in the 25 µg/mL and 50 µg/mL groups, respectively.

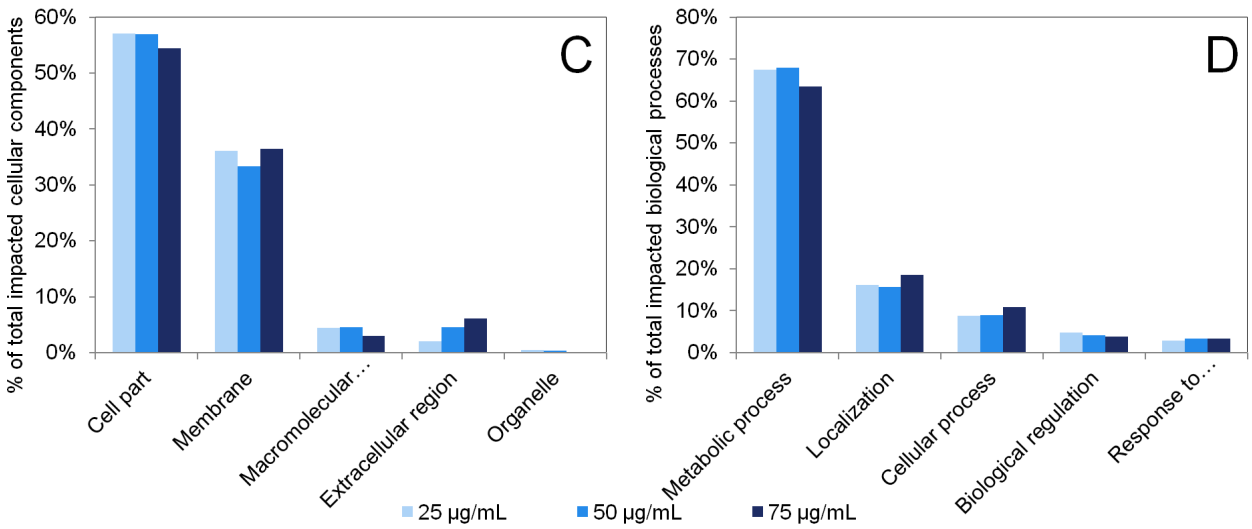


Figure 21. Impacted cellular components (C) and biological processes (D) from the differentially expressed genes. Regarding cellular components, no trends were observed in cell part, membrane, macromolecular complex, or organelles. An increase in extracellular region was observed. In terms of impacted biological processes, no trends were noted as a function of drug concentration, including on response to stimulus.

Out of 68 impacted pathways by the differential gene expression, Figure 22 shows the 10 most affected. Only three of these pathways show trends that suggest a correlation with Gentamicin Sulfate concentration: an increase in chorismate biosynthesis and in *de novo* pyrmidine ribonucleotides biosynthesis, and a decrease in folate biosynthesis. The increase in chorismate is likely due to the under-expression of the *aro* genes in the 25 µg/mL samples, while they increasingly overexpressed in the 50 and 75 µg/mL groups; the same phenomenon, but with the *pyr* and *car* genes, explains the trend in *de novo* pyrmidine ribonucleotides biosynthesis. The main driver behind the changes in folate biosynthesis is the *folE* gene's expression.

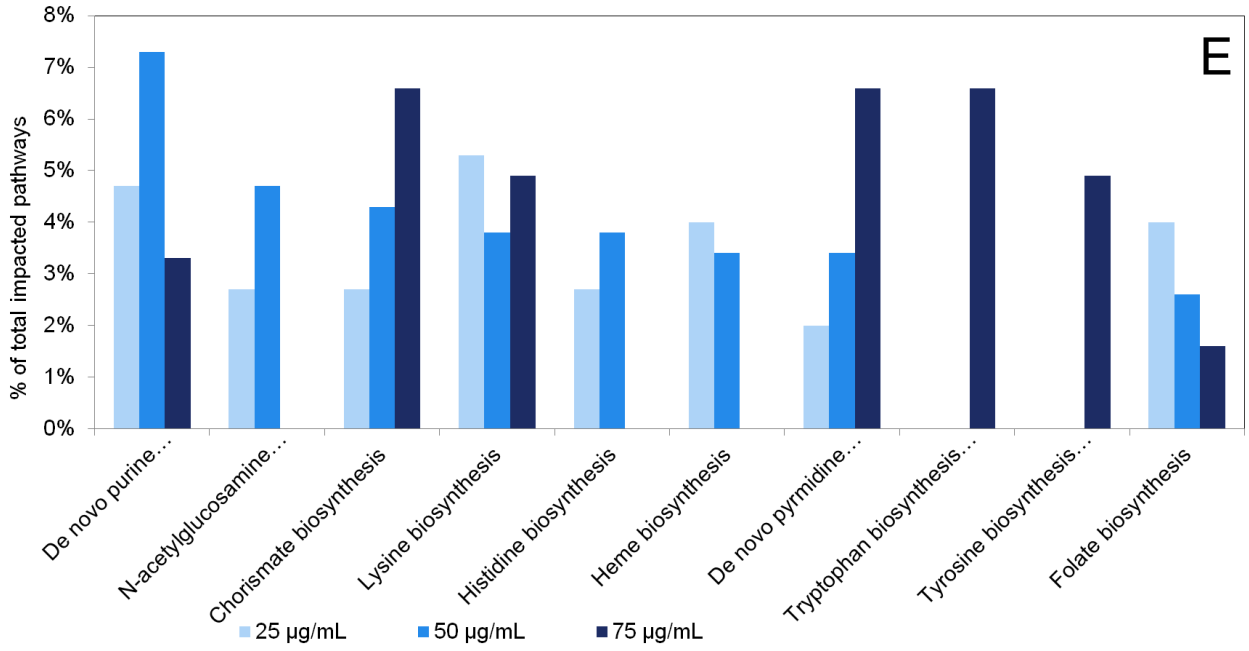


Figure 22. Impacted pathways by the differential gene expression. No trends were observed as a function of drug concentration on *de novo* purine biosynthesis, N-acetylglucosamine metabolism, or in tryptophan or tyrosine biosynthesis. An increase was noted in chorismate biosynthesis and *de novo* pyrmidine ribonucleotides biosynthesis. There was a decrease in folate biosynthesis.

6.5 GENES ASSOCIATED WITH RESISTANCE TO AMINOGLYCOSIDES

A literature survey was conducted to assemble a list of genes that have been associated with resistance to aminoglycosides; Table 9 describes them and their respective fold in/decrease in the AES-1 samples. From the 31 genes identified, only six were observed to be differentially expressed in the spaceflight samples, three being up-regulated and the other three showing both up- and down-regulation. The only gene showing some kind of trend is *OppA*, which was up-regulated in two of the three scenarios. However, *OppA* has been associated with resistance to aminoglycosides when it is *under*expressed, as it assists in the transport of the molecule through the cellular membrane (Acosta et al., 2000).

Table 9. List of genes associated with resistance to aminoglycoside antibiotics.

Gene name	Function	25 µg/mL	50 µg/mL	75 µg/mL	Reference on resistance
aaD2	Uncharacterized	-	-	-	(Magnet & Blanchard, 2005)
AcrA	Acriflavine resistance protein A	-2.22	2.09	-	(Elkins & Nikaido, 2002)
AcrB	Acriflavine resistance protein B	-2.03	2.15	-	(Magnet & Blanchard, 2005)
AcrD	Probable aminoglycoside efflux pump	-	-	-	(Magnet & Blanchard, 2005) (Elkins & Nikaido, 2002)
adeB	Uncharacterized	-	-	-	(Magnet & Blanchard, 2005)
adeRS	Uncharacterized	-	-	-	(Magnet & Blanchard, 2005)
amrR69	Uncharacterized	-	-	-	(Magnet & Blanchard, 2005)
ecfB	Uncharacterized	-	-	-	(Thorbjarnardáttir, Magnúsdóttir, Eggertsson, Kagan, & Andrésson, 1978)
emrR	Uncharacterized	-	-	-	(Xiong et al., 2000)
GmrA	Uncharacterized	-	-	-	(Magnet & Blanchard, 2005)
kanA	Uncharacterized	-	-	-	(Thorbjarnardáttir et al., 1978)
KgmB	Uncharacterized	-	-	-	(Magnet & Blanchard, 2005)
KsgA	Uncharacterized	-	2.42	-	(O'Farrell, Scarsdale, & Rife, 2004)
ksgC	Uncharacterized	-	-	-	(Yoshikawa, Okuyama, & Tanaka, 1975)
Mar	Uncharacterized	-	-	-	(May, Ito, & Okabe, 2009)
MdfA	Multidrug transporter MdfA	-	-	-	(Magnet & Blanchard, 2005)
mexZ	Uncharacterized	-	-	-	(Magnet & Blanchard, 2005)
nfr	Uncharacterized	-	-	-	(Morozov, Nosova, Biketov, Valiaev, & Domaradski□, 1994)
OppA	Periplasmic oligopeptide-binding protein	-	5.87	2.81	(Acosta et al., 2000)
purE	N5-carboxyaminoimidazole ribonucleotide mutase	-2.19	4.77	-	(Yoshikawa et al., 1975)
rpsL	30S ribosomal protein S12	-	-	-	(Gill & Amyes, 2004)
rpxE	Uncharacterized	-	-	-	(De Wilde, Cabezón, Villarroel, Herzog, & Bollen, 1975)
rpxL	Uncharacterized	-	-	-	(De Wilde et al., 1975)
sir	Uncharacterized	-	-	-	(Delcuve, Cabezon, Herzog, Cannon, & Bollen, 1978)
Sox	Uncharacterized	-	-	-	(May et al., 2009)
strA	Uncharacterized	-	-	-	(Delcuve et al., 1978)
Suc	Uncharacterized	-	-	-	(Collis & Grigg, 1989)
TolC	Outer membrane protein TolC	-	3.96	-	(May et al., 2009)
unc	Uncharacterized	-	-	-	(Thorbjarnardáttir et al., 1978)
YhcQ	Uncharacterized	-	-	-	(May et al., 2009)
yigQR	Uncharacterized	-	-	-	(Macinga, Cook, Poole, & Rather, 1998)

6.6 GENES ASSOCIATED WITH OXIDATIVE STRESS

A list of 48 *E. coli* genes associated with defense from oxidative stress was acquired from (Farr & Kogoma, 1991) and presented in Table 10. The samples challenged with 25 µg/mL showed an up-regulation in six genes and a down-regulation in 9 genes. While only

one gene was overexpressed (and none were underexpressed) in the 75 µg/mL group, 27 were up-regulated and none were down-regulated in the 50 µg/mL set. The *wrbA* gene outstands for its high-fold expression increase and because it was overexpressed in two different drug concentration scenarios.

Table 10. List of genes associated with oxidative stress and their fold in/decrease.

Gene	25 µg/mL	50 µg/mL	75 µg/mL	Gene	25 µg/mL	50 µg/mL	75 µg/mL
ahpC	-	-	-	*nth*	-	-	-
ahpF	-	-	-	*ompF*	-	9.84	5.47
apaH	-	2.15	-	*oxyR*	-3.41	2.18	-
appA	9.46	4.23	-	*oxyS*	-	-	-
arcA	-2.58	-	-	*polA*	-	3.47	-
dam	-2.71	2.57	-	*polC*	-	-	-
dnaK	7.57	3.25	-	*recA*	-	-	-
gor	-	2.44	-	*recB*	-2.45	-	-
groE	-	-	-	*recC*	-5.61	-	-
groL	5.22	3.42	-	*rimK*	-	-	-
groS	4.62	3.44	-	*rpoH*	-	-	-
grx	-	-	-	*sodA*	-	5.47	-
gshA	-	2.43	-	*sodB*	-	3.13	-
gyrB	-	-	-	*soi*	-	-	-
katE	-	2.43	-	*sox*	-	-	-
katF	-	-	-	*topA*	-	2.58	-
katG	-	3.55	-	*trxA*	-	3.22	-
ksgA	-	2.42	-	*trxB*	-3.06	2.77	-
micF	-	-	-	*uvrA*	-	-	-
mutM	3.89	2.35	-	*uvrB*	-	-	-
mvrA	-	-	-	*uvrC*	-2.55	2.61	-
ndh	-	5.65	-	*wrbA*	22.71	7.66	-
nfo	-3	2.77	-	*xthA*	-4.25	2.33	-
nrdB	-	2.25	-	*zwf*	-	2.9	-

6.7 GENES ASSOCIATED WITH ACID RESISTANCE

Weber et al. (2005) presented a list of eleven σ^S-regulated genes associated with acid resistance, nine of which were overexpressed (in between 2.01x and 29.08x) and none was under-expressed in the AES-1 samples, as seen in Table 11. It is noteworthy that, again, only one gene was overexpressed in the 75 µg/mL group. Nevertheless, up-regulation can be seen on all the interrogated genes except two (*hdeD* and *yhiU)* in the two other scenarios.

Table 11. List of σ^S-regulated genes associated with acid resistance and their fold increase.

Gene	25 µg/mL	50 µg/mL	75 µg/mL
gadA	7.82	11.11	-
gadB	5.75	23.04	-
gadC	8.96	5.69	-
gadE	20.62	5.82	-
gadW	6.04	28.09	-
gadX	7.21	29.08	-
hdeA	4.49	14.86	2.01
hdeB	4.78	9.52	-
hdeD	-	-	-
slp	9.84	-	-
yhiU	-	-	-

A comprehensive list of genes associated with acid resistance, regardless of the regulator, was reported in (Tucker et al., 2002), from which 24 other genes were identified, and listed on Table 12. From the 24 genes, 15 were overexpressed in the AES-1 samples. One of them, *yccJ* was the gene overexpressed the most (26.63x) in the 25 µg/mL samples. Only one gene was underexpressed (in the 25 µg/mL group); however, it was overexpressed in the 50 µg/mL scenario.

Table 12. List of non-σ^S-regulated genes associated with acid resistance and their fold in/decrease.

Gene	25 µg/mL	50 µg/mL	75 µg/mL	Gene	25 µg/mL	50 µg/mL	75 µg/mL
asr	-	9.75	-	*ydiZ*	2.42	4.58	3.11
cbpA	2.55	3.7	-	*yeaQ*	7.04	6.26	2.83
cfa	-	-	-	*yebV*	-	3.71	-
dps	4.78	3.6	-	*yfbE*	-	-	-
ompC	-2.21	2.8	-	*yfbF*	-	-	-
osmY	-	3.23	-	*ygfR*	-	-	-
wrbA	22.71	7.66	-	*yhiE*	-	-	-
yahO	-	-	-	*yhiF*	4.1	6.29	-
ybaS	6.95	2.62	-	*yhiM*	6.89	6.94	-
ybaT	3.74	-	-	*yhiW*	-	-	-
ycaC	6.37	5.92	-	*yiaG*	11.34	8.45	2.94
yccJ	26.63	7.22	-	*yifC*	-	-	-

Table 11 and Table 12 show that, from a total of 35 genes associated with acid resistance, 24 were up-regulated (in between 2.01x and 29.08x). This data suggest that the cells indeed were under acidic stress.

6.8 SUMMARY

The per-drug analysis showed that 28 genes were commonly up-regulated throughout all three test sets. These genes include the *mal* operon, which transports maltose and other substrates across cellular membranes; and the *thi* genes, which were the highest up-regulated genes in this set and which are involved with catalytic activity and several metabolic processes. The list of 28 genes also includes several from the *opp* group, which transport oligopeptides (sources of carbon) but also aminoglycosides into the cell. Several *suc* genes were also overexpressed among all the three assessed test scenarios (25, 50 and 75 μg/mL); they are involved with succinyl-Co-A processes, and Suc^- phenotypes of *E. coli* have shown cross-resistance to aminoglycosides. Only one gene, *cusF*, associated with cation efflux system, was found to be commonly under-expressed throughout all the three test sets.

The per-scenario approach allowed for an analysis to be conducted individually for the three drug concentration data sets. The 25 μg/mL samples showed an up-regulation of the *gadE* and *yccJ* genes, both associated with conferring *E. coli* with acid resistance. The *ecnA* gene, the antidote to the *ecnB* toxin gene, was the single most underexpressed gene in this set; *ecnAB* is an antidote/toxin gene pair (also known as addiction molecules) that control apoptosis, or programmed cell death during starvation conditions. The *wrbA* gene, believed to have a role in oxidative stress defense and/or cell signaling (and which is also acid-induced), was up-regulated. The 50 μg/mL samples showed an up-regulation on the whole *trp* operon, which is associated with the formation of amino acids and organic acids –

the *trp* were the five genes that incremented in expression the most. Overexpression of the *gadABC* and *E* genes, and of *hdeA*, and *B*, all associated with acid resistance, was noted. Another set of genes that were up-regulated in this group were *malE* and *malK*, which are involved in the transport of all substrates through the inner cell membrane. The *thi* genes, associated with thiamine biosynthetic enzymes needed for carbohydrate metabolism, were overexpressed. None of the 12 down-regulated genes was reduced by a factor smaller than -5x. The 75 µg/mL samples exhibited overexpression of the *trp* and *mal* genes, previously discussed.

A literature survey was conducted to assemble a list of 31 genes that have been associated with resistance to aminoglycosides, from which only six were differentially expressed and only one was *over*expressed in more than one of the three antibiotic concentration scenarios. This was the *oppA* gene, which permits bacteria to resist aminoglycosides when it is *under*expressed, the opposite of what was observed on the spaceflight samples.

A similar analysis took place where the overexpressed genes in the AES-1 spaceflight samples were checked against a list of 48 genes associated with oxidative stress responses. The 25 µg/mL samples showed an up-regulation in six and a down-regulation in nine; while only one gene was overexpressed (and none were underexpressed) in the 75 µg/mL group, However, 27 were up-regulated and none were down-regulated in the 50 µg/mL set.

Finally, another review indicated that there are 35 genes associated with acid resistance in *E. coli*, from which 24 were up-regulated (in between 2.01x and 29.08x) in the AES-1 spaceflight samples.

6.9 DISCUSSION

Table 13 associates the individual results of the preliminary gene expression analysis with observations relevant to the AES-1 experiment, and categorizes them in four groups: acid response, lysis, metabolism, and transport.

Table 13. AES-1 differentially expressed genes of special interest and their fold in/decrease. Genes with a white background are associated with acid response; those in a light brown background are associated with lysis; in light blue with metabolic processes, and in dark gray with trans-membrane transport.

Gene	25 µg/mL	50 µg/mL	75 µg/mL	Gene	25 µg/mL	50 µg/mL	75 µg/mL
asr	-	9.75	-	*yhiW*	-	-	-
cbpA	2.55	3.7	-	*yiaG*	11.34	8.45	2.94
cfa	-	-	-	*yifC*	-	-	-
dps	4.78	3.6	-	*ecnA*	-34.31	-	-
gadA	7.82	17.49	-	*ecnB*	15.7	3	-
gadB	5.75	25.6	-	*aceE*	-	3.42	-
gadC	8.96	11.11	-	*aceF*	-	3.57	-
gadE	20.62	23.04	-	*lpd*	-	2.91	-
gadW	6.04	5.69	-	*pfl*	-	-	-
gadX	7.21	5.82	-	*poxB*	4.7	3.48	-
hdeA	4.49	28.09	-	*sucA*	2.7	4.07	2.11
hdeB	4.78	29.08	-	*sucB*	3.91	3.6	2.05
hdeD	-	14.86	2.01	*sucC*	3.33	4.16	2.22
hdeE	-	-	-	*sucD*	4.22	4.39	2.29
ompC	-2.21	2.8	-	*thiE*	2.84	28.59	4.29
osmY	-	3.23	-	*thiF*	3	28.87	4.93
slp	9.84	9.52	-	*thiG*	2.34	30.48	5.32
wrbA	22.71	7.66	-	*thiH*	2.37	24.88	5.06
yahO	-	-	-	*thiS*	3	32.41	3.97
ybaS	6.95	2.62	-	*trpA*	-	69.11	12.46
ybaT	3.74	-	-	*trpB*	-	53.43	13.16
ycaC	6.37	5.92	-	*trpC*	-	45.61	14.06
yccJ	26.63	7.22	-	*trpD*	-	60.78	19.45
ydiZ	2.42	4.58	3.11	*trpE*	-	46.74	22.29
yeaQ	7.04	6.26	2.83	*cusF*	-	-	-
yebV	-	3.71	-	*malE*	2.81	43.8	24.09
yfbE	-	-	-	*malK*	-	22.39	24.28
yfbF	-	-	-	*oppA*	-	5.87	2.81
ygfR	-	-	-	*oppB*	2.67	4.68	2.63
yhiE	-	-	-	*oppC*	3	4.3	2.52
yhiF	4.1	6.29	-	*oppD*	4.36	4.27	2.6
yhiM	6.89	6.94	-	*oppF*	4.08	4.77	2.73
yhiU	-	-	-	*yhiW*	-	-	-

6.9.1 Resistance to Aminoglycosides

From the 31 genes know to confer *E. coli* with resistance to aminoglycosides (e.g. Gentamicin Sulfate), only six were differentially expressed in space. From this list, the *oppA* gene was the only up-regulated gene in more than one of the three AES-1 samples sets. However, this resistance is conferred to *E. coli* when the gene is *under*expressed, as this gene transports the antibiotic into the cell, but it was *over*expressed in the spaceflight cultures, enabling more antibiotic molecules to permeate through the cellular membrane. **The results of the gene expression analysis suggest that the currently known resistance mechanisms against *E. coli* were not systematically activated**. This might be an indication that, if *E. coli* became more resistant to Gentamicin Sulfate in space, it did so via a mechanism not yet characterized. However, it is argued that for a novel mechanism to be activated, the cell would have needed to be challenged with a concentration of antibiotic that would have likely up-regulated some of the genes already known to help *E. coli* resist the drug. Because this up-regulation was not observed, this data suggests that **drug molecules may have reached the cell at a lower rate, thus eliciting increased bacterial growth with respect to the ground controls**.

6.9.2 Acid Response

The gene expression analysis conducted on these AES-1 samples shows that, from the 35 genes known to be induced by acid conditions, 24 were overexpressed in the spaceflight cultures. **This indicates that there was an increase in acidity in the (intra- and/or extra-) cellular environment.** Tucker et al. (2002) characterized 26 acid-induced genes and concluded that 13 of them were up-regulated by acetate: (*cfa, dps, gadA, gadB, hdeA, hdeB, hdeD, ompC, osmY, slp, yccJ, yeaQ,* and *yhiX*). Ten of these genes were overexpressed in the AES-1 spaceflight samples, suggesting that **the increase in acidity may have**

been in part due to a rise in acetate concentration in and/or around the cell. The acetate connection is investigated further in the Metabolism paragraph.

6.9.3 Role of the *rpoS* gene and σ^S

The *rpoS* gene encodes the sigma factor σ^S (RpoS protein) when the cell is under environmental stress to regulate the transcription of 156 genes that may permit the bacteria to survive (Weber, Polen, Heuveling, Wendisch, & Hengge, 2005). Although σ^S is the master regulator of the general stress response in *E. coli* (Weber et al., 2005), no trends were observed on the spaceflight samples in terms of *rpoS* differential expression. An underexpression (-3.48x) was observed in the 25 µg/mL samples and no changes were present in the two other sets. Nevertheless, of eleven σ^S-regulated acid response-related genes, nine were up-regulated in the spaceflight samples. However, it was reported that these genes' dependence on RpoS is reduced or even abolished under acid stress conditions (Weber et al., 2005). **The gene expression data indicates that there was no overexpression of the *rpoS* gene, which is usually activated by environmental stress. An analysis of the complete gene expression data set, and of the 156 genes regulated by RpoS, will be needed to arrive to conclusive results.**

6.9.4 Apoptosis

The *ecnAB* is an antidote/toxin gene pair that was differentially expressed in the spaceflight samples. The toxin gene *ecnB* was up-regulated in two of the three sets (between 3.00x and 15.70x) while the antidote gene *ecnA* was down-regulated (-34.31x) in the first group. The *ecnAB* gene pair is encoded by the enterocidin locus, a chromosomal bacteriolytic module of *E. coli* that is regulated by osmotic signals during starvation at stationary phase, and programs the cell to die, likely to provide nutrients to remaining

healthy cells in the population (Bishop, Leskiw, Hodges, Kay, & Weiner, 1998). **This indicates that there likely were 1) osmolarity differences, and 2) an increased starvation condition in the spaceflight samples with respect to their matched ground controls.** It is of interest that, although the enterocidin locus is regulated by RpoS, no systematic overexpression of the *rpoS* gene was observed. This might indicate that exposure to spaceflight may have exposed a novel regulator of the *ecnAB* suicide genes. Currently, these genes cannot be artificially up-regulated to cause bacterial cells to die via RpoS because this protein also up-regulates several stress-response mechanisms (Weber et al., 2005). However, **if cultures grown in space could show a novel regulation mechanism of the enterocidin locus, this could be exploited to kill drug-resistant bacteria on Earth**.

6.9.5 Oxidative Stress

The conflicting results between the 25 μg/mL (where six genes were up-regulated and nine were down-regulated), 50 μg/mL (where 27 genes were up-regulated), and 75 μg/mL (where only one gene was overexpressed), out of 48 genes associated with oxidative stress response, sets **makes it difficult to arrive to a conclusion in terms of potential oxidative stress in the spaceflight samples**. Seven genes were overexpressed in two drug concentration sets, from which *wrbA* outstands for the high fold of its up-regulation (22.71x and 7.66x). This gene synthesizes the WrbA flavoprotein, which is believed to have a role in oxidative stress defense and/or cell signaling (Kishko et al., 2012). The *oxyR* gene, associated with peroxide stress response in *E. coli* and *Salmonella typhimurium*, was down-regulated in the 25 μg/mL samples (-3.41x), up-regulated in the 50 μg/mL samples (2.18x), and non-differentially expressed in the 75 μg/mL group. **The overexpression of *oxyR* in**

only one of the three sets (and at a low fold) suggests that, if the cells were under oxidative stress, this was likely not coming from peroxide molecules.

6.9.6 Metabolism

To investigate the potential acetate connection further, the genes related with acetate production were interrogated. Three enzymes convert pyruvate to acetyl compounds: pyruvate oxidase PoxB (*poxB*), pyruvate dehydrogenase PDH (*aceEF* and *lpd*), and pyruvate formate lyase Pfl (*pfl*), where the encoding gene is in parenthesis (Chang, Wang, & Cronan, 1994); as seen in Table 13, all of these genes were up-regulated in the AES-1 spaceflight samples. When conducting a similar assessment on the other potential metabolic byproducts, it was noted that virtually all of the genes characterized as involved in these metabolic pathways were overexpressed in the spaceflight samples (Figure 23). **This suggests that overall metabolic activity was stimulated in space, which could be involved with the observed increase in bacterial proliferation in microgravity.**

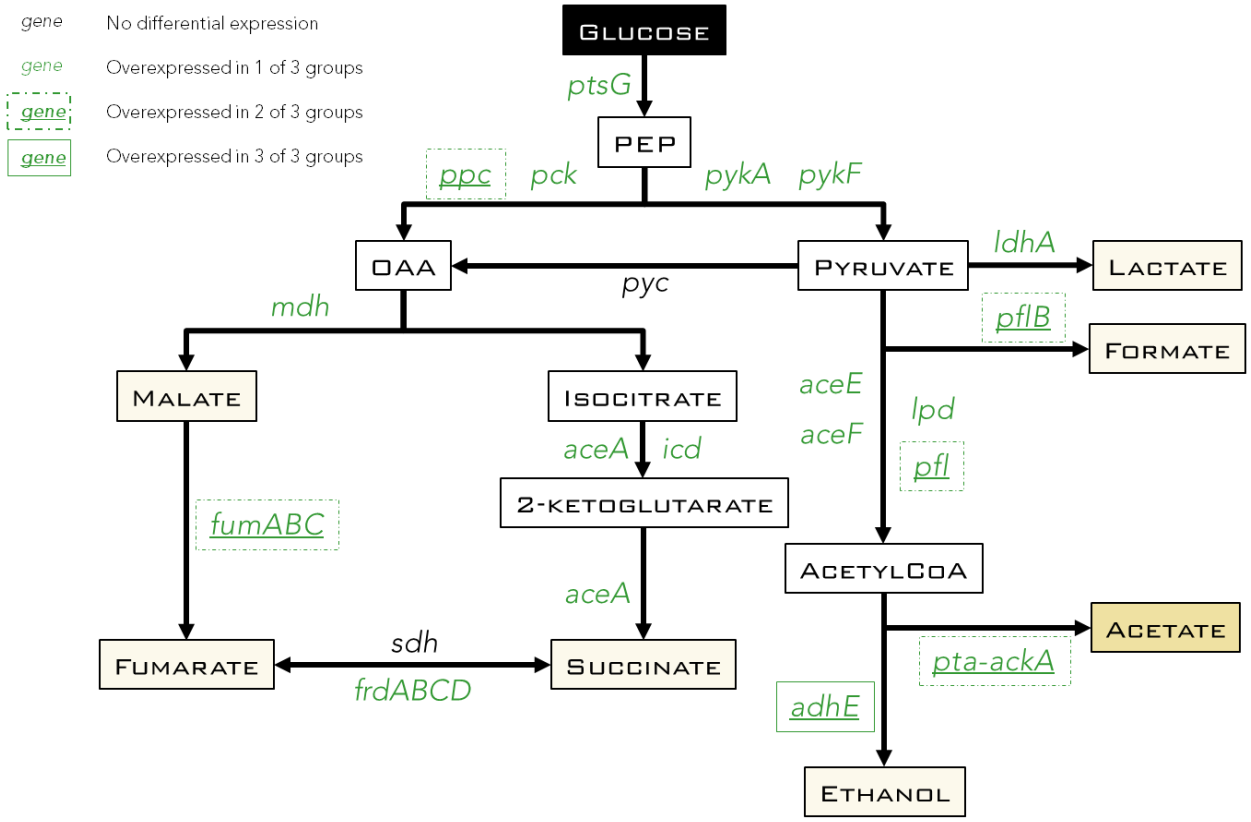


Figure 23. *E. coli* metabolic pathways, compiled from (Liu & Jarboe, 2012) and (Chang et al., 1994). Most of the genes associated with these metabolic pathways were overexpressed in the spaceflight samples with respect to the ground controls.

The up-regulation of the *trp* operon, which is associated with the formation of amino acids and organic acids, may be related with the processes shown in Figure 23 although a clear correlation has not yet been found. In general, the *trp* operon is regulated by the presence of tryptophan in the environment through a negative feedback loop, which suggests that there was a decrease in environmental tryptophan for the *trp* operon to be so overexpressed (up to 69.11x). The *malE* gene was also up-regulated in all three sets; however, the encoding of the MalE protein is under a positive control, meaning that its synthesis is inducible by maltose (Kellermann & Szmelcman, 1974). In the AES-1 experiment the source of carbon for *E. coli* was glucose ($C_6H_{12}O_6$) and not maltose ($C_{12}H_{22}O_{11}$), so an explanation to this phenomenon still needs to be formulated. The *thi*

genes were also overexpressed in all of the data sets. They synthesize thiamine, which is needed for carbohydrate metabolism. Although further research is needed to corroborate this, the overexpression of the *thi, opp,* and *mal* genes may suggest that this was a response to carbon starvation as *E. coli* accumulates adenosine thiamine triphosphate (AThTP), a form of thiamine, in response to lack of energy substrates (Gigliobianco et al., 2010). The *suc* genes, which are also associated with metabolism, were also upregulated; their role in the overall observations is still not clear. **This information suggests that 1) a stimulation of several metabolic pathways increased the production of conjugate bases such as acetate, and formate; and 2) the cells were under a carbon starvation environment in space.**

6.9.7 Other Studies

Because gene expression data has not yet been produced for the cultures challenged with higher concentrations of Gentamicin Sulfate, (100-175 μg/mL) a study to correlate cell aggregation to gene expression has not yet been conducted. Another investigation that may be worth undertaking when the complete gene expression data package is received, is to assess if there is a correlation between the differential regulation of genes associated with cell signaling (for quorum sensing), e.g. *wrbA*, and the phenotypic observations.

CHAPTER 7

CONCLUSIONS

The literature survey on spaceflight investigations in which antibiotic activity and cell envelope thickness were assessed, presented in Chapter 4, shows that few results have been reported with a supporting statistical analysis and have been free of potential confounding from re-adaptation to 1*g*. From the ones that have, it can be concluded that non-motile *E.coli* has shown increased growth with respect to ground controls, both in liquid medium and agar, and has proliferated in 1XMIC of Gentamicin in liquid medium. On the other hand, it has also been documented that the same drug showed a 10% increase in effectiveness on the same bacterial strain when tested in agar. This stresses the importance of the role that growth medium (liquid vs. solid) play on the extracellular environment. This literature review also shows that several scientific questions remain unanswered, such as if there are changes on antibiotic activity or binding, or on bacterial cellular envelope due to gravity; or if fluid behavior plays a role on bacterial susceptibility to antibiotics in space; or the differentiation between the roles of microgravity and radiation on the observed phenomena in space. Finally, this thorough literature survey and analysis demonstrates that there are yet no sound conclusions regarding changes on bacterial cell envelope due to spaceflight conditions.

The conclusions from the Antibiotic Effectiveness in Space (AES-1) experiment, presented in Chapter 5, are summarized and presented in Table 14 together with information regarding their respective statistical analyses and their results. One-way ANOVA were conducted to assess the role of drug concentration on a) cell length and b) diameter for the four different combinations of drug type (Gentamicin Sulfate and Colistin Sulfate) and gravity environment (spaceflight and ground control). There were no statistically significant differences in either condition, suggesting that cell size is independent of drug concentration. Therefore, samples were grouped in spaceflight *vs*. ground controls and paired student t tests were conducted.

Table 14. Synopsis of differences on spaceflight cultures challenged with Gentamicin Sulfate (except "Cell concentration at time of antibiotic introduction", where cultures were antibiotic-free) with respect to matched ground controls, and their paired student t test.

Parameter	Conclusion	Result
Cell concentration at time of antibiotic introduction	No statistically significant difference	M_{space} = 6.91x10^6 *cell/mL* SD_{space} = 7.98x10^6*cell/mL*; M_{ground} = 7.88x10^6 *cell/mL*, SD_{ground} = 1.65x10^6 *cell/mL* (t(10) = -0.24, p = 0.8151)
Cell Length	Statistically significant: Cells in space were **71%** the length of ground controls	M_{space} = 1.660 µm, SD_{space} = 0.288 µm; M_{ground} = 2.353 µm, SD_{ground} = 0.603 µm (t(310) = -2.07, p = 0.0389)
Cell Diameter	Statistically significant: Cells in space were **78%** the length of ground controls	M_{space} = 0.627 µm, SD_{space} = 0.084 µm; M_{ground} = 0.809 µm, SD_{ground} = 0.131 µm (t(310) = -2.33, p = 0.0203)
Cell Volume	Statistically significant: Cells in space had **41%** the volume of ground controls	M_{space} = 0.457 µm^3, SD_{space} = 0.155 µm^3; M_{ground} = 1.109 µm^3, SD_{ground} = 0.534 µm^3 (t(310) = -2.35, p = 0.0196)
Cell Surface	Statistically significant: Cells in space had **54%** the volume of ground controls	M_{space} = 3.281 µm^2, SD_{space} = 0.762 µm^2; M_{ground} = 6.021 µm^2, SD_{ground} = 2.027 µm^2 (t(310) = -2.53, p = 0.0119)
Cell Envelope Thickness	Not enough data produced yet	
Antibiotic Effectiveness – 25 µg/mL Gentamicin	Statistically significant: **7-fold increase** in final cell concentration in space with respect to ground controls	M_{space} = 4.38x10^8 *cell/mL*, SD_{space} =2.87x10^7 *cell/mL*; M_{ground} = 6.19x10^7 *cell/mL*, SD_{ground} = 5.01x10^7 *cell/mL* (t(5) = 13.03, p = 0.00005)
Antibiotic Effectiveness – 50 µg/mL Gentamicin	Statistically significant: **41-fold increase** in final cell concentration in space with respect to ground controls	M_{space} = 4.87x10^8 *cell/mL*, SD_{space} = 4.54x10^7 *cell/mL*; M_{ground} = 1.18x10^7 *cell/mL*, SD_{ground} = 1.59x10^7 *cell/mL* (t(5) = 19.75, p = 0.00001)
Antibiotic Effectiveness – 75 µg/mL Gentamicin	Statistically significant: **18-fold increase** in final cell concentration in space with respect to ground controls	M_{space} = 3.10x10^8 *cell/mL*, SD_{space} = 1.01x10^8 *cell/mL*; M_{ground} = 1.71x10^7 *cell/mL*, SD_{ground} = 1.12x10^7 *cell/mL* (t(6) = 5.74, p =0.00121)
Antibiotic Effectiveness – Overall Gentamicin	Statistically significant: **13-fold increase** in final cell concentration in space wrt ground controls	M_{space} = 4.01x10^8 *cell/mL*, SD_{space} = 1.03x10^8 *cell/mL*; M_{ground} = 3.03x10^7 *cell/mL*, SD_{ground} = 3.66x10^7 *cell/mL* (t(20) = 6.77, p =0.000001)

The preliminary assessment of gene differential expression was conducted first in a per-drug fashion, where the spaceflight data was compared to that of the ground controls, and in a per-scenario (i.e. per drug concentration) approach, where flight and matched ground controls were compared in three sets: 25, 50, and 75 μg/mL (since that is the gene expression data available at this time). The per-drug analysis showed that 28 genes were commonly up-regulated throughout all three test sets. These genes include the *mal* operon, which transports maltose and other substrates across cellular membranes; and the *thi* genes, which were the highest up-regulated genes in this set and which are involved with catalytic activity and several metabolic processes. The list of 28 genes also includes several from the *opp* group, which transport oligopeptides (sources of carbon) but also aminoglycosides into the cell. Several *suc* genes were also overexpressed among all the three assessed test scenarios (25, 50 and 75 μg/mL); they are involved with succinyl-Co-A processes, and Suc^- phenotypes of *E. coli* have shown cross-resistance to aminoglycosides. Only one gene, *cusF*, associated with cation efflux system, was found to be commonly under-expressed throughout all the three test sets.

The per-scenario approach allowed for an analysis to be conducted individually for the three drug concentration data sets. The 25 μg/mL samples showed an up-regulation of the *gadE* and *yccJ* genes, both associated with conferring *E. coli* with acid resistance. The *ecnA* gene, the antidote to the *ecnB* toxin gene, was the single most underexpressed gene in this set. *ecnAB* is an antidote/toxin gene pair (also known as addiction molecules) that control apoptosis, or programmed cell death during starvation conditions. The *wrbA* gene, believed to have a role in oxidative stress defense and/or cell signaling (and which is also acid-induced), was up-regulated. The 50 μg/mL samples showed an up-regulation on the whole *trp* operon, which is associated with the formation of amino acids and organic acids – the *trp* were the five genes that incremented in expression the most. Overexpression of the

gadABC and *E* genes, and of *hdeA*, and *B*, all associated with acid resistance, was noted. Another set of genes that were up-regulated in this group were *malE* and *malK*, which are involved in the transport of all substrates through the inner cell membrane. The *thi* genes, associated with thiamine biosynthetic enzymes needed for carbohydrate metabolism, were overexpressed. None of the 12 down-regulated genes was reduced by a factor smaller than -5x. The 75 µg/mL samples exhibited overexpression of the *trp* and *mal* genes, previously discussed.

A literature survey was conducted to assemble a list of 31 genes that have been associated with resistance to aminoglycosides, from which only six were differentially expressed and only one was *over*expressed in more than one of the three antibiotic concentration scenarios. This was the *oppA* gene, which permits bacteria to resist aminoglycosides when it is *under*expressed, the opposite of what was observed on the spaceflight samples. It was concluded that the genes associated with conferring resistance to the aminoglycoside class of antibiotics to *E. coli* were not systematically activated, as shown in Figure 24.

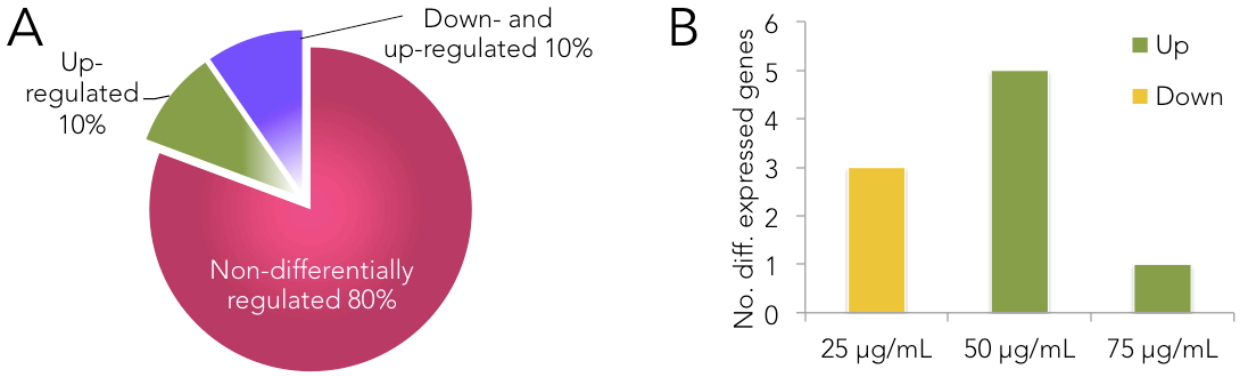


Figure 24. Differential expression of the 31 genes associated with conferring resistance to the aminoglycosides class of antibiotics in terms of general distribution (A) and per-scenario (B).

A similar analysis took place where the overexpressed genes in the AES-1 spaceflight samples were checked against a list of 48 genes associated with oxidative stress

responses. As seen in Figure 25, the 25 µg/mL samples showed an up-regulation in six and a down-regulation in nine; while only one gene was overexpressed (and none were underexpressed) in the 75 µg/mL group, However, 27 were up-regulated and none were down-regulated in the 50 µg/mL set. No conclusions could be reached in terms of oxidative stress due to the inconsistency in gene expression results on the three scenarios.

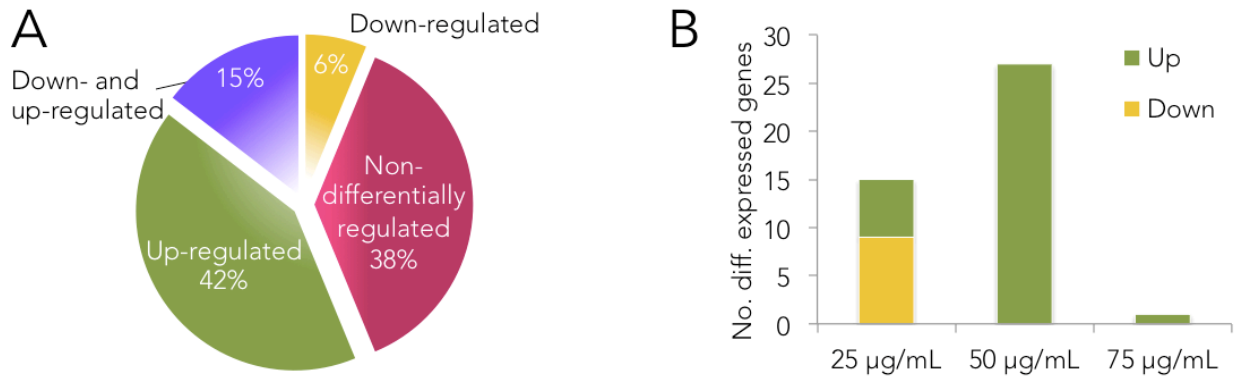


Figure 25. Differential expression of the 48 genes associated with conferring resistance to the oxidative stress in terms of general distribution (A) and per-scenario (B).

Finally, another review indicated that there are 35 genes associated with acid resistance in *E. coli*, from which 24 were up-regulated (in between 2.01x and 29.08x) in the AES-1 spaceflight samples, as seen in Figure 26. This up-regulation was observed in all three scenarios. From this, it was concluded that there was an increase in acidity in the (intra- and/or extra-) cellular environment. Furthermore, 10 out of 13 genes up-regulated in the presence of high concentrations of acetate were overexpressed, as seen in Figure 26 (C). Similarly, 9 out of 11 genes that confer resistance to acidity and which are regulated by RpoS were overexpressed, despite of the fact that *rpoS* was not up-regulated. This is also indication of the increase in acidity around the cell because these genes' dependence on RpoS is reduced or even abolished under acid stress conditions.

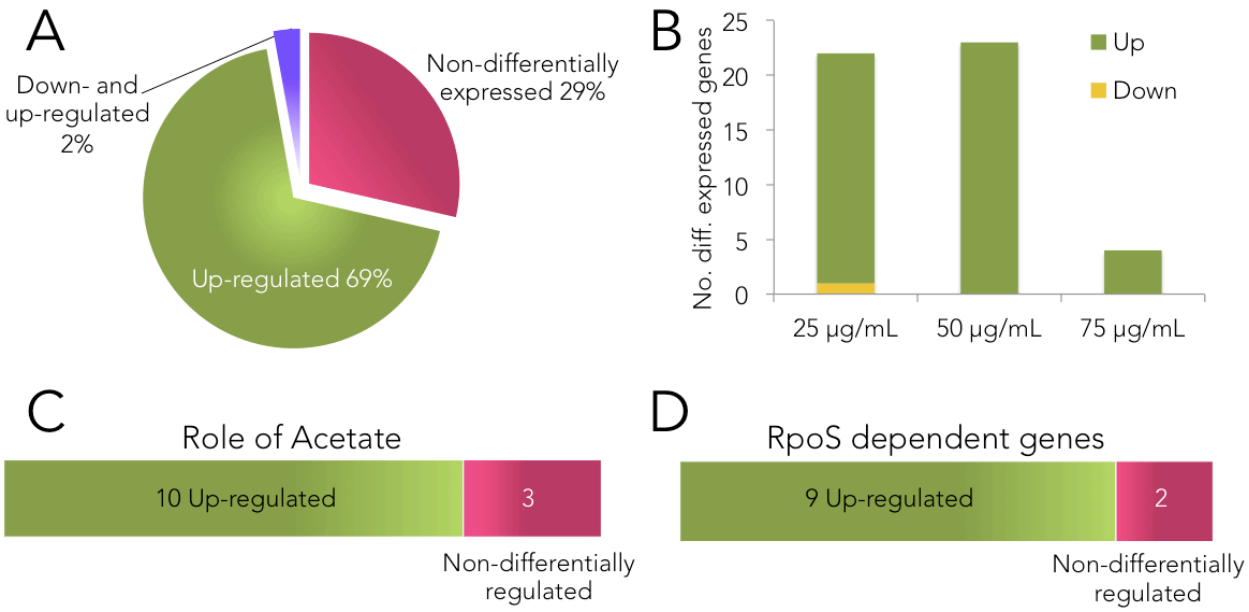


Figure 26. Differential expression of the 35 genes associated with acid resistance in terms of general distribution (A) and per-scenario (B). (C) shows that 10 out of 13 genes that are up-regulated by acetate were overexpressed. Similarly, 9 out of 11 RpoS-dependent genes that confer acid resistance under high-acidity environments, but that abolish their dependence on RpoS under extreme conditions (*rpoS* was not up-regulated), were up-regulated (D).

The most important conclusions from the gene expression data analysis are summarized in

Table 15.

Table 15. Summary of conclusions from the gene expression observed in space with respect to the matched ground controls.

Parameter	Conclusion	Supporting data
Drug-resistance	No systematic activation of the genes known to confer resistance (resistome) to aminoglycosides	Only 6 out of 31 associated genes were overexpressed. Gene that confers *sensitivity* to antibiotics (*oppA*) was up-regulated
Extra-cellular environment: **Acidity**	There was an increase in **acidity** the intra- and/or extra-cellular environment	24 out of 35 associated genes were up-regulated
		9 out of 11 genes that are regulated by RpoS, but are regulated even when *rpoS* is not activated (which was the case) if there are high-acidity conditions
	There was an increase in **acetate** concentration in the intra- and/or extra-cellular environment	10 out of 13 associated genes were up-regulated
Role of *rpoS* (σ^S)	No overexpression of the stress-master regulator *rpoS*	Only differential expression was (-3.48x) in the 25 µg/mL samples
Apoptosis	The RpoS-regulated **suicide gene** was overexpressed without *rpoS* being activated. This suggests there might be a novel mechanism to activate the suicide process in (drug-resistant) bacteria.	The toxin gene *ecnB* was up-regulated in two of the three sets (3.00x, 15.70x); the antidote gene *ecnA* was down-regulated (-34.31x) in the first group, their known regulator, *rpoS*, was not activated.
Oxidative Stress	No conclusion regarding oxidative environment reached	Results show similar trends in down-, up-, and non-differentially gene expression
	Peroxide concentration was not increased in spaceflight cultures	No systematic overexpression of *oxyR*
Metabolism	Overall metabolic activity was stimulated in space	19 out of 21 glucose catalysis-associated genes were up-regulated
Extra-cellular environment: **Nutrients (Glucose)**	This data suggests that cells were under carbon starvation	Up-regulation of genes associated with the transport of substrates into the cell, accumulation of AthTP during starvation, and 19 out of 21 glucose-catalysis associated genes

CHAPTER 8

DISCUSSION

A literature survey on antibiotic effectiveness in space shows that the questions that were posed at the dawn of this research field remain unanswered: “are there changes in antibiotic activity in space?” and “are there changes in cellular envelope thickness in space?” Additionally, the first seven spaceflight experiments to address these issues have posed other questions, such as “does fluid behavior play a role on bacterial susceptibility to antibiotics in space?”. Figure 4, in Chapter 4, shows how has our understanding of this field progressed during the last decades and serves to define the state of knowledge prior to the Antibiotic Effectiveness in Space (AES-1) experiment.

Conclusions from AES-1 include that *E. coli* was able to survive in concentrations of Gentamicin Sulfate that on Earth would be inhibitory, and that the spaceflight samples grew to a smaller cell size, and showed a tendency to aggregate when challenged with high concentrations of Gentamicin. In addition to these phenotypic changes, the results from the gene expression analysis suggest that the currently known resistance mechanisms against *E. coli* were not systematically activated. Because this up-regulation was not observed, this data suggests that drug molecules may have reached the cell at a lower rate, thus eliciting increased bacterial growth with respect to the ground controls. The gene expression

analysis also indicates that there was an increase in acidity in the (intra- and/or extra-) cellular environment; this increase in acidity may have been in part due to a rise in acetate concentration.

The gene expression data indicates that there was no overexpression of the *rpoS* gene, which is usually activated by environmental stress. An analysis of the complete gene expression data set, and of the 156 genes regulated by RpoS, will be needed to arrive to conclusive results. The overexpression of *ecnB* and underexpression of *ecnA* indicates that there likely were 1) osmolarity differences, and 2) an increased starvation condition in the spaceflight samples with respect to their matched ground controls.

Conflicting results in term of potential oxidative stress makes it difficult to arrive to a conclusion. However, the overexpression of *oxyR* in only one of the three sets (and at a low fold) suggests that, if the cells were under oxidative stress, this was likely not coming from peroxide molecules.

The differential gene expression analysis suggests that overall metabolic activity was stimulated in space, which could be involved with the observed increase in bacterial proliferation in microgravity. This study suggests that 1) a stimulation of several metabolic pathways increased the production of conjugate bases such as acetate, and formate; and 2) the cells were under a carbon starvation environment in space.

8.1 PROPOSED MODEL

Figure 27 graphically describes a working theory of what occurs in the extracellular environment in space (A and C) compared to 1g (B and D) that could explain some of the observed phenomena; this is only a graphic representation – a not-yet validated theoretical computer model has been published in (Klaus et al., 2004)). Changes in the extracellular

fluid environment can be summarized in four different scenarios: in these images, red indicates high concentration, while blue represents low concentration of a given chemical component. Under 1g (B and D), there is density-driven fluid motion as well as cell sedimentation, both gravity-driven. On the other hand, in microgravity (A and C), cell motion is provided only through active means (e.g. flagella) and particle displacement through Brownian motion, as explained in (Klaus et al., 2004). In the case of AES-1, where the bacterial model was non-motile, no active cell movement is assumed.

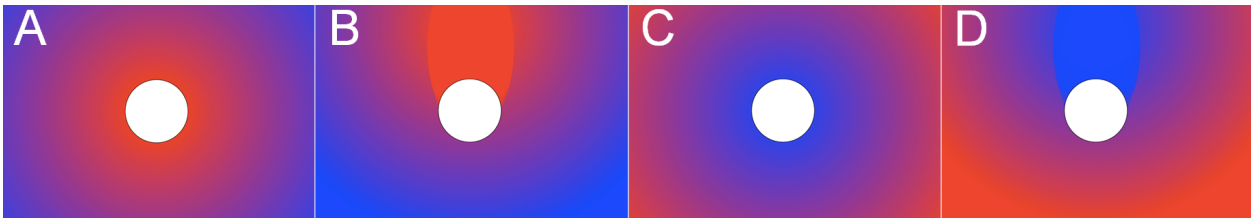


Figure 27. Graphical models of altered extracellular fluid environment. Red indicates high concentration of a chemical component and blue is a low concentration. The white circle is a simplification of a bacterial cell in the fluid. (A) and (C) represent the microgravity environment while (B) and (D) the 1g environment, where a cell sediments.

The results of the gene expression analysis can then be correlated with these four potential extracellular fluid environments explanations, as described in Table 16.

Table 16. Potential extracellular fluid environment explanations to the observed differential gene expression. Up-arrow indicates up-regulation; down arrow down-regulation.

Hypothetical (H) or measured (M) phenotypic result in space	Associated Molecule	Potential extracellular fluid environment explanation		Gene expression supporting extracellular environment explanation
		Spaceflight	Ground Controls	
(M) Increased bacterial growth when challenged with drugs	Antibiotic	C	D	No systematic activation of *E.coli*'s known resistome to aminoglycosides
(H) Increased acidity around cell	Carboxylic Acids	A	B	1) ↑24 out of 35 acid-reponse genes, 2) ↑9 out of 11 rpo-s dependent acid-response genes activated despite lack of rpoS overexpression, 3) ↑10 out of 13 acetate-production genes, 4) ↑5 out of 5 acetyl compound-producing genes
(H) Starvation	Glucose	C	D	*1)* ↑*ecnB,* ↓*ecnA, 2)* ↑*malE,* ↑*malK, 3)* ↑*thi*
(H) Increased metabolic activity	Glucose	C	D	1) ↑19 out 21 glucose metabolism-genes, 2) ↑5 out of 5 acetyl compound-production genes
(H) Decrease in environmental tryptophan	Tryptophan	C	D	↑*trp*
(H) Increased trans-membrane transport (starvation related?)	Several	C	D	*1)* ↑*malE,* ↑*malK, 2)* ↑oppA, 3) ↓cusF
(H) Change in environmental osmolarity (?)	Unknown			↑*ecnB,* ↓*ecnA,* although *rpoS* was not overexpressed
(M) Decrease in cell size				Not yet clarified
(M) Cell aggregation				Not yet clarified

Figure 28 summarizes the hypothesized processes that could explain the observed phenomena in space. Microgravity triggers alterations in the extracellular environment, in this case, reducing the concentration of glucose molecules around the cell (as in Figure 27-C). This produces a starvation environment for the bacterial cells, which up-regulate their metabolic processes to synthesize substrates. This would explain the observed increase in trans-membrane transport gene expression and the observed bacterial proliferation in

spaceflight (regardless if challenged or no with antibiotics). The up-regulated metabolism then increases the production of carboxylic acids creating a region of high acidity around the cell (as in Figure 27-A) since they move away from the cell exclusively by Brownian motion. This, in turn, up-regulates the genes that confer *E. coli* with acid resistance. It is not yet clear what the consequences of this overexpression may be.

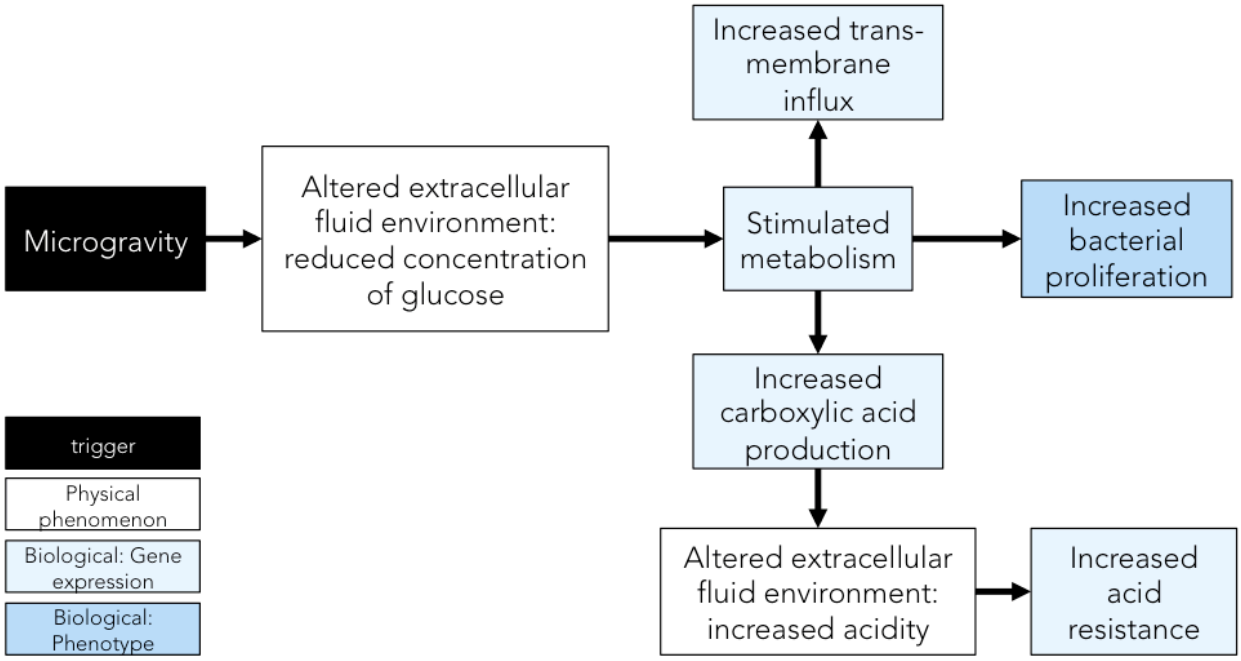


Figure 28. Hypothesized process that explains the observed increased bacterial proliferation in space (regardless if cells were challenged with antibiotics).

Figure 29 depicts the hypothesis that explains the observed bacterial proliferation in concentrations of drugs that on Earth are inhibitory. The fact that there was no systemic up-regulation (some genes were even down-regulated in space) suggest that there was a reduced concentration of antibiotics around the cell.

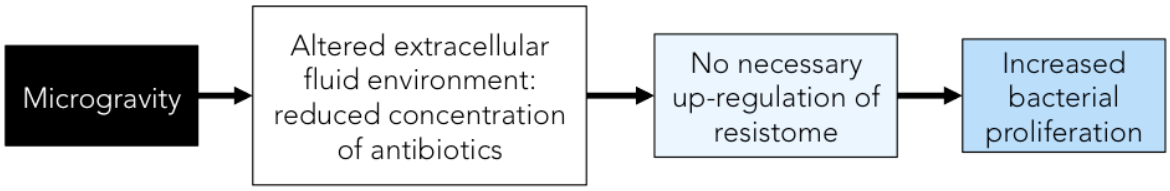


Figure 29. Hypothesized process that explains the observed bacterial proliferation in concentrations of antibiotics that are inhibitory on Earth.

Figure 30 shows the pathway for the activation of the entericidin locus (also known as a "suicide module" because it causes the cell to program its own death) and its eventual up-regulation of the toxin gene *ecnB* and down-regulation of the antidote gene *ecnA*, (differential expression of *ecnAB* was observed in the spaceflight samples). The activation of the *rpoS* gene is necessary, as it synthesizes the RpoS protein that, in turn, activates the entericidin locus. However, *rpoS* showed no differential expression in two of the three space culture sets and was underexpressed in the third. This poses the question of what caused the up-regulation of *ecnB* (up to 15.7x) and down-regulation of *ecnA* (down to -34.31x).

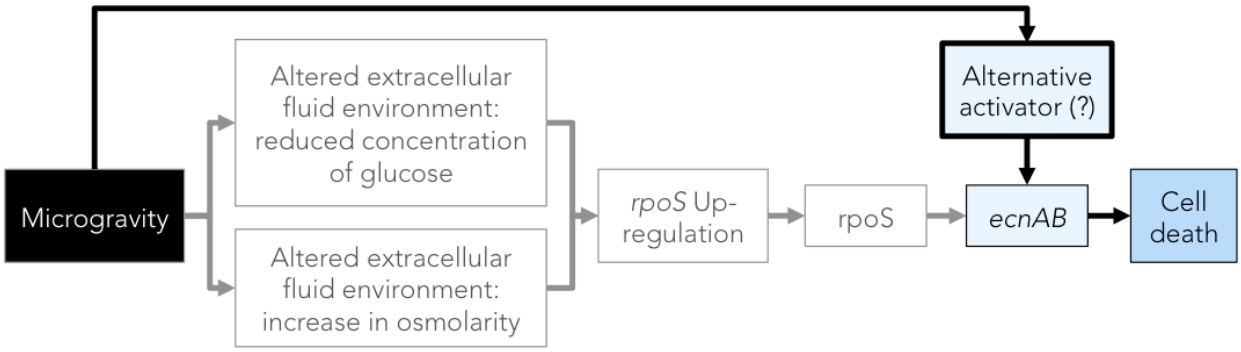


Figure 30. The activation of the entericidin locus and eventual cell death. No overexpression of the *rpoS* gene was observed, so it is not yet clear what caused the activation of *ecnAB*. Learning of an alternative activator of this locus, which might have been up-regulated in spaceflight, could serve as a novel drug target.

Because *rpoS* (the known activator of the entericidn locus) was not overexpressed, but the locus was differentially expressed, it is believed that there is an alternative activator of the entericidin locus that was turned on during spaceflight. This alternative activator could potentially be used as a novel antibiotic target. The activation of the *ecnB* gene is of interest because it programs cell death by synthesizing lipoproteins and eventually causing lysis (Bishop et al., 1998). This occurs naturally when *rpoS* is activated by starvation and increase osmolarity. However, if the entiricidin locus could be activated via an antibiotic molecule, cells could be "re-programmed" to kill themselves, which could prove beneficial against drug-resistant bacteria.

The description of the evolution of knowledge in these matters, originally presented in Figure 4, is updated in Figure 31 with the conclusion and hypothesis derived from this thesis work. Additionally, Table 17 summarizes the conclusions regarding each hypothesis. For the first one, it was found that this is generally true with the caveat that antibiotic efficacy is not the correct term, as that was not the measured parameter. The better explanation is that, in space, a lower rate of antibiotics reached each bacterial cell.

Table 17. Conclusions reached about each hypothesis. "G" speaks of Gentamicin Sulfate, while "C" of Colistin Sulfate.

Hypothesis	Conclusion
Antibiotics used to treat bacteria grown in space will exhibit reduced efficacy and will be associated with specific changes in bacterial gene expression that correlate with cell survival	See above explanation
G1/C1: When challenged with "G"/"C" in microgravity, *E. coli* ATCC 4157 cells will grow to larger sizes compared to matched 1g controls	False
G2/C2: When challenged with "G"/"C" in microgravity, *E. coli* ATCC 4157 cells will develop thicker cell envelopes compared to matched 1g controls	Undetermined
G3/C3: When challenged with "G"/"C" in microgravity, *E. coli* ATCC 4157 cells will grow to higher final cell concentrations compared to matched 1g controls	True
G4/C4: When challenged with "G"/"C" in microgravity, *E. coli* cells ATCC 4157 will have reduced lag phases compared to matched 1g controls	Undetermined
G5/C5: In microgravity, *E. coli* ATCC 4157 will proliferate under normal (1g) inhibitory concentrations of "G"/"C"	True
G6/C6: There is a correlation between population growth dynamics, cell size, and cell envelope thickness of *E. coli* ATCC 4157, and bacterial susceptibility to "G"/"C"	True

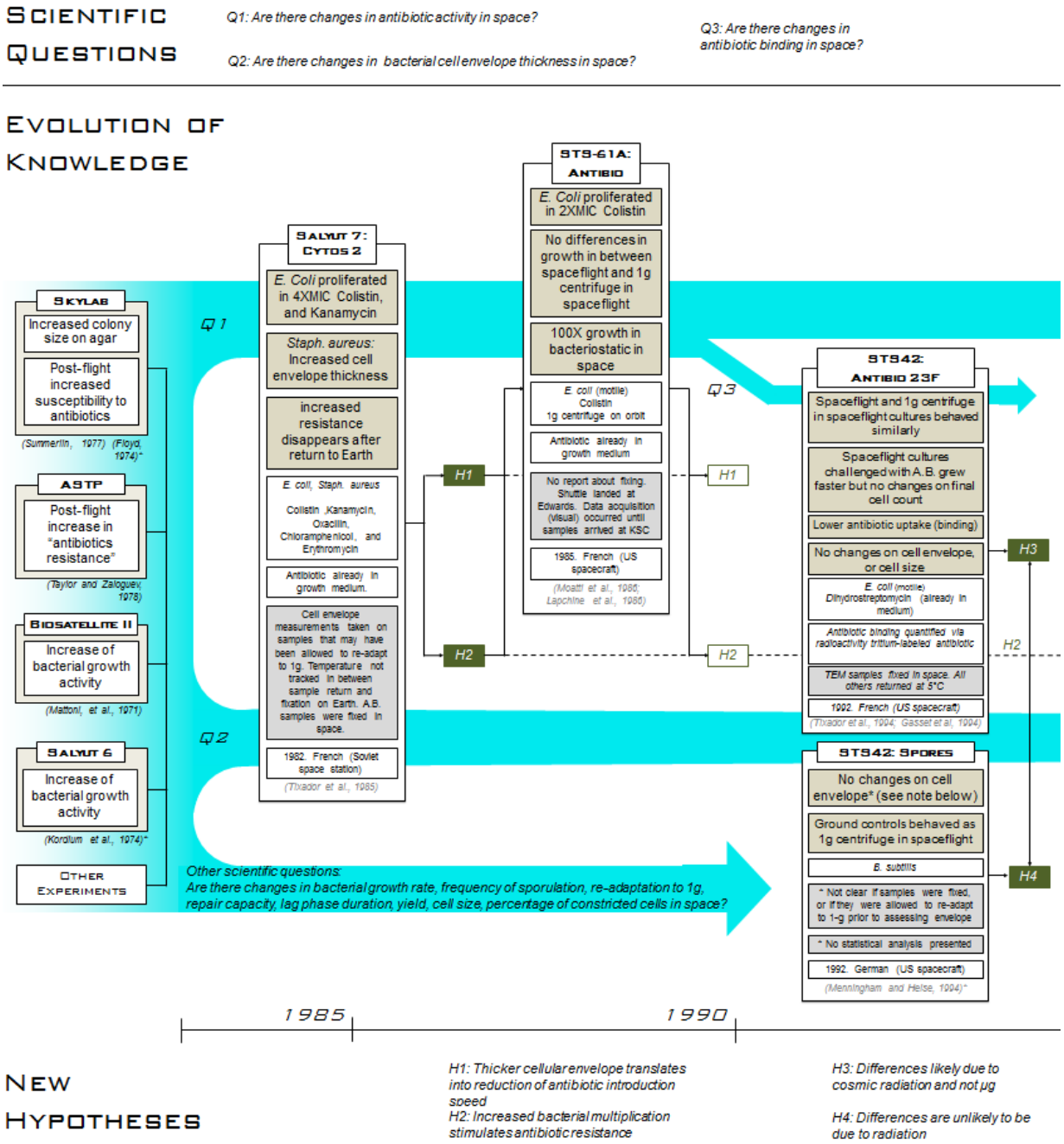
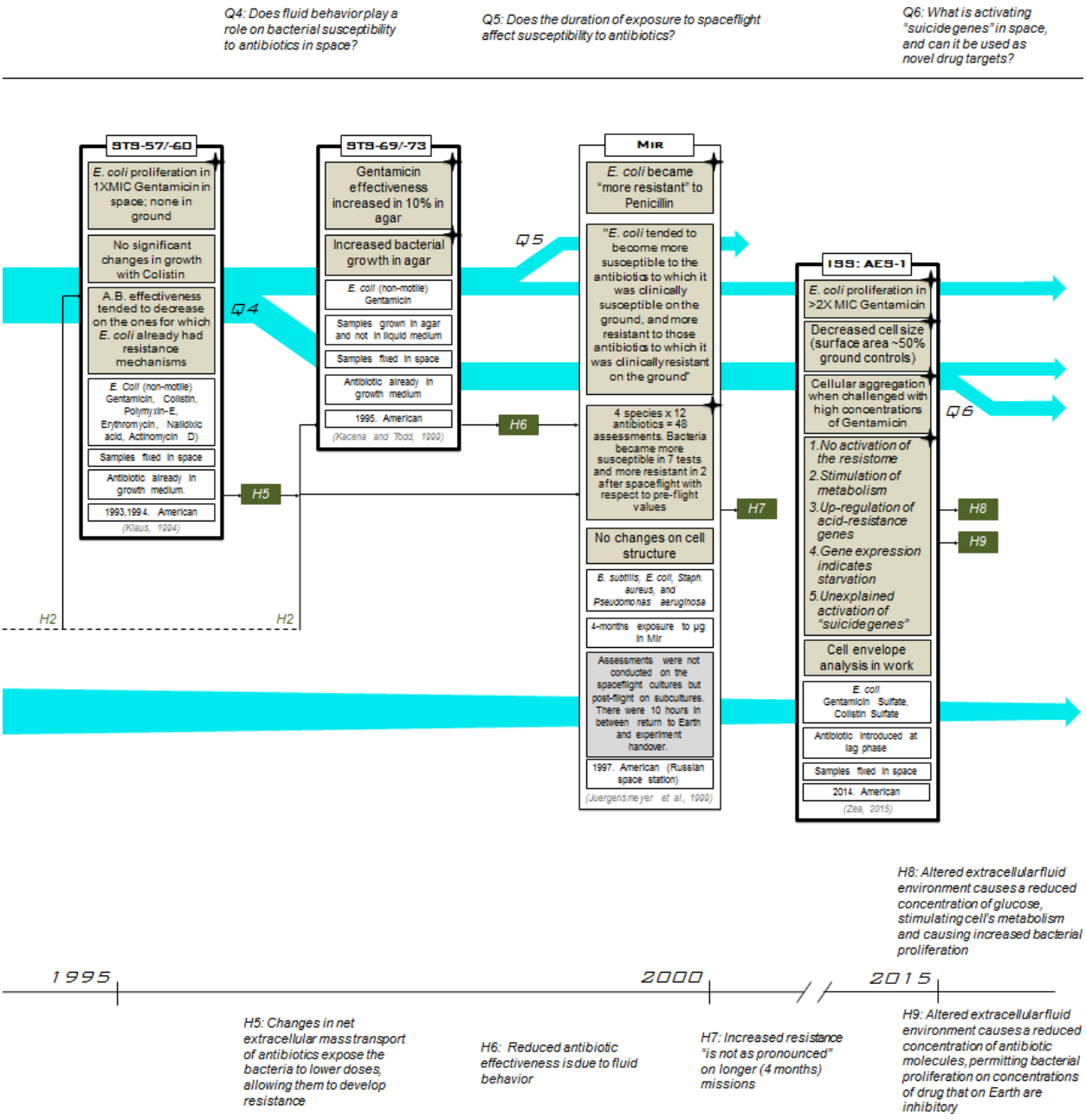


Figure 31. Evolution of knowledge diagram with conclusion and hypotheses derived from this thesis work.


Q4: Does fluid behavior play a role on bacterial susceptibility to antibiotics in space?
Q5: Does the duration of exposure to spaceflight affect susceptibility to antibiotics?
Q6: What is activating "suicide genes" in space, and can it be used as novel drug targets?
STS-57/-60
E. coli proliferation in 1XMIC Gentamicin in space; none in ground
No significant changes in growth with Colistin
A.B. effectiveness tended to decrease on the ones for which E. coli already had resistance mechanisms
E. Coli (non-motile) Gentamicin, Colistin, Polymyxin-E, Erythromycin, Nalidixic acid, Actinomycin D)
Samples fixed in space
Antibiotic already in growth medium.
1993,1994. American
(Klaus, 1994)
STS-69/-73
Gentamicin effectiveness increased in 10% in agar
Increased bacterial growth in agar
E. coli (non-motile) Gentamicin
Samples grown in agar and not in liquid medium
Samples fixed in space
Antibiotic already in growth medium
1995. American
(Kacena and Todd, 1999)
MIR
E. coli became "more resistant" to Penicillin
"E. coli tended to become more susceptible to the antibiotics to which it was clinically susceptible on the ground, and more resistant to those antibiotics to which it was clinically resistant on the ground"
4 species x 12 antibiotics = 48 assessments. Bacteria became more susceptible in 7 tests and more resistant in 2 after spaceflight with respect to pre-flight values
No changes on cell structure
B. subtilis, E. coli, Staph. aureus, and Pseudomonas aeruginosa
4-months exposure to µg in Mir
Assessments were not conducted on the spaceflight cultures but post-flight on subcultures. There were 10 hours in between return to Earth and experiment handover.
1997. American (Russian space station)
(Juergensmeyer et al., 1999)
ISS: AES-1
E. coli proliferation in >2X MIC Gentamicin
Decreased cell size (surface area ~50% ground controls)
Cellular aggregation when challenged with high concentrations of Gentamicin
1. No activation of the resistome
2. Stimulation of metabolism
3. Up-regulation of acid-resistance genes
4. Gene expression indicates starvation
5. Unexplained activation of "suicide genes"
Cell envelope analysis in work
E. coli Gentamicin Sulfate, Colistin Sulfate
Antibiotic introduced at lag phase
Samples fixed in space
2014. American
(Zea, 2015)
Q4
Q5
Q6
H2
H2
H5
H6
H7
H8
H9
H8: Altered extracellular fluid environment causes a reduced concentration of glucose, stimulating cell's metabolism and causing increased bacterial proliferation
1995
2000
2015
H5: Changes in net extracellular mass transport of antibiotics expose the bacteria to lower doses, allowing them to develop resistance
H6: Reduced antibiotic effectiveness is due to fluid behavior
H7: Increased resistance "is not as pronounced" on longer (4 months) missions
H9: Altered extracellular fluid environment causes a reduced concentration of antibiotic molecules, permitting bacterial proliferation on concentrations of drug that on Earth are inhibitory

8.2 THESIS CONTRIBUTIONS

This thesis' contributions can be summarized as follows:

1. Reproduced previous spaceflight results that bacteria were able to grow in inhibitory levels of antibiotic.
   - Validation of earlier observations presented in (Tixador et al., 1985; Moatti et al., 1986; Lapchine et al., 1986; Tixador et al., 1994; Gasset et al, 1994; (Menningham and Heise, 1994; Klaus, 1994; Kacena and Todd, 1999; Juergensmeyer et al., 1999).
2. Provided data that corroborates the extracellular environment model
   - Stimulated acid response "almost proves" the theory that byproducts accumulate in the extracellular fluid environment presented in (Klaus, 1994).
   - Up until now, this had proved unachievable through physical (Owen et al., 2002), and computational modeling (Benoit et al., 2008), but is clarified via overexpressed genes associated with acid resistance (this work).
3. Observed that *E. coli* cell size was decreased in space
   - This unexpected smaller cell size observed in space cultures translates into smaller cell surface area which reduces target size for antibiotics.
4. Characterized stimulated metabolism may explain in part why bacteria tend to grow better in space
   - Also suggests potential means for improving bioengineering processes
5. Gene expression data suggests that reduced bacterial susceptibility to antibiotics in space is not due to increased drug resistance (a microbial defense response), rather due to a decrease in antibiotic molecule concentration around the cell (a microgravity biophysical mass transport effect).

6. The up-regulation of suicide genes in space without their regulator being overexpressed may present a novel target against drug-resistant pathogens on Earth.

CHAPTER 9

RECOMMENDATIONS

9.1 FOR INVESTIGATORS STARTING SPACE-BASED RESEARCH

Spaceflight places challenges on research that are best addressed during early experiment design. It is recommended to use low-biosafety level organisms (eases the flight safety-related processes) and, depending on the scientific objectives, to keep bacterial motility as a strain selection criterion. Similarly, it is suggested to use non-toxic, and non-hazardous materials to the extent possible (again, to keep safety-related processes as simple as possible). The experiment should be planned to minimize the impact of the delay between sample preparation and experiment start (due to payload integration into the spacecraft/launch vehicle, potential launch delays, initiation of operations after reaching space, etc.). This can be achieved by maintaining the organisms in stasis, either by temperature (if power and temperature regulation are available to the organism habitat), or by maintaining it in a medium without a source of glucose or metabolic energy, if possible. Given the limitation on up-mass, it is also recommended to prioritize sample replicate number over amount of testing conditions to enable statistically significant results.

Ideally, scientific assays should be conducted in space; however, this is difficult to achieve. To avoid potential re-adaption of the organism to 1*g*, the next best solution is to fix

the samples in space as soon as the experiment has been completed. Researchers should also keep in mind what assays and protocols will be conducted on Earth and choose their fixative accordingly. Researchers should consider the use of a 1*g* centrifuge in orbit to assess the roles of gravity and radiation on their dependent variables. The use of standardized protocols are recommended to make the spaceflight results available and compatible to other investigations on Earth.

9.2 FOR NEXT RESEARCH STEPS

It is encouraged that cellular envelope investigations are continued, as it has been proved that changes on the bacterial cell envelope are correlated with antibiotic resistance here on Earth (Sieradzki & Tomasz, 2003), and a literature review and analysis showed that conclusive data hasn't yet been produced. These new investigations should include -omics types of analyses, e.g. transcriptomics and genomics, to elucidate the mechanisms behind the observed changes in space. It is also recommended that the role of fluid behavior be closely studied by the use of liquid medium and agar in a parallel and synchronous fashion.

The analysis conducted on the AES-1 experiment identified correlations between gene expression and phenotypic expression. Future studies should test these correlations to find actual causation and the different governing mechanisms behind the observed decreased susceptibility to antibiotics in space.

When the complete data set of differential gene expression is available, a study to correlate cell aggregation to gene expression should be conducted. Another investigation that may be worth undertaking at that time is to assess if there is a correlation between the differential regulation of genes associated with cell signaling (for quorum sensing), e.g. *wrbA*, and the phenotypic observations.

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APPENDIX 1 - *E. COLI* GROWTH PROCEDURE

The most basic experimental activity related to this thesis was the growth of bacteria in the laboratory. Some of the common denominators throughout the experiments are:

- Incubated at 30°C.
- *E. coli* grown in 1X Medium E with 5 g/L glucose as growth medium.

There are four steps to the growth of bacteria:

A. Preparation of growth media

B. Inoculation

C. Growth of individual cultures and concentration measurements

D. Data analysis

Preparation of Growth Medium

Medium E at a 50X concentration, described by (Vogel & Bonner, 1956), must be prepared in a clean beaker as per Table 18.

Table 18. ME minimal growth medium constituents. Constituents and their required amounts to produce 100 mL of 50X Medium E (Vogel & Bonner, 1956).

Component	Amount
$MgSO_4 \cdot 7H_2O$	1.0 g
Citric Acid $\cdot H_2O$	10.0 g
$K_2HPO_4 \cdot anhydrous$	50.0 g
$NaNH_4(HPO_4) \cdot 4H_2O$	17.5 g
Distilled Water	67 ml

The amounts described above of each of the constituents produce 100 mL of 50X Medium E. For mixing, it is recommended to use a magnetic stirrer, as the 50X solution is highly viscous. The salinity of this highly concentrated solution impedes bacterial growth, thus there is no need for sterilization at this stage. However, 0.1 mL of chloroform can be added to the 100mL of solution as a preservative. During the experiments presented in this thesis no chloroform was used.

In a separate, clean container, a 5 g/L solution of glucose in distilled water is prepared. This solution is used to dilute the 50X Medium E solution to a 1X concentration. The amount of glucose solution needed to conduct this dilution can be calculated from the dilution equation:

$$C_1V_1 = C_2V_2$$

Where C is concentration, V is volume and the 1 and 2 subindexes differentiate two different states of the solution. For example, if 250 mL of 1X Medium E wants to be produced, then the amount of 50X solution can be calculated as follows:

$$V_1 = \frac{C_2}{C_1}V_2 = \frac{1}{50} \cdot 250\ \text{mL} = 5.0\ \text{mL}$$

This means that the final solution will consist of 5 mL of 50X Medium E and 245 mL of dilutant solution. The amount of glucose is calculated based on the total, final volume, i.e. 250 mL:

$$\left(5\ {}^{\text{g}}/_{\text{L}}\right)(.250\ \text{L}) = 1.25\ \text{g}$$

At this point there are two different vessels: one containing 5 mL of 50X Medium E and a second with 1.25g of glucose in 245 mL of distilled water. These two containers are then autoclaved and finally, mixed. Manual stirring for two or three minutes is enough to achieve a homogeneous solution.

Several growth media will be tested for *B. subtilis* and *S. aureus*. A growth medium will be selected based on the criteria: 1) ensure non-motility and certainty of metabolic byproducts and 2) provide a growth rate that permits clear distinctions between the phases of growth. Both of these bacteria will be tested in Medium E, produced as detailed in this chapter. *B. subtilis* will also be grown in M9 minimal medium as in (Fuhrer, Fischer, & Sauer, 2005; Tännler, Decasper, & Sauer, 2008).

Inoculation

Several screw-capped, 9 mL Pyrex® 9825 test tubes were used for individual cultures. If the bacteria were originating in a solid state, a medium transition batch would be grown to allow the cells to adapt to the new medium. Otherwise, the test tube containing the bacteria in 1X Medium E, here referred to as "Continuity batch", would be stirred until appearing homogeneous and placed in an incubator **at 30°C** for 12 hours. Actually, the Continuity batch was always maintained at that temperature and refreshed with Medium E as necessary. Then, a small amount (<0.5 mL) of the continuity batch would be transferred into a 9.0 mL test tube and then this container would be filled to the top with 1X Medium E – this is referred to as the "Grandmother Culture". The date, time and temperature would then be recorded and the test tube labeled accordingly. All cultures were maintained in an incubator to control the temperature to avoid the introduction of other confounding factors.

Growth of individual cultures and concentration measurements

When the Grandmother culture reached its mid-exponential phase ($OD_{600} \approx 0.3$ or ~12 hours)(Benoit et al., 2008), its vessel was stirred until the content was homogeneous in color. Then, a sample was taken from it to be placed on a hemocytometer on the microscope and/or place in a 96 well, flat bottom, plate in a Spectrophotometer ran at 600 nm. If using a Spectrophotometer, liquid was filled until the surface was flushed to the well's top, trying to minimize surface concavity/convexity. If the concentration in the grandmother culture was measured with a hemocytometer, then this was accomplished by counting the number of cells in five different quadrants of the hemocytometer as seen in Figure 32.

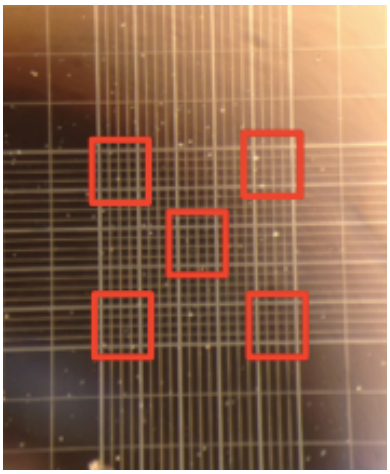

Figure 32. Used quadrants in hemacytometer.

Each of the quadrants in a red square is 0.2 mm in side and 0.100 mm in depth, thus containing a volume of 4.0×10^{-6}mL. This volume allows to calculate the concentration in the solution: each cell observed within this quadrant is then equivalent to a $2.5\times10^{5}\ {}^{\text{cell}}/_{\text{mL}}$.

If a Spectrophotometer was used, the Grandmother culture's cell concentration was calculated with the an OD vs. cell concentration equation developed from data points acquired in the lab following the procedure indicated in this chapter. After the

concentration in the mother culture had been either measured or calculated, the series of cultures can start to be prepared. For this purpose, the following equations are used

Number of individual cultures to be grown: a

Volume of each culture: $b\ (mL)$

Total amount of volume needed c where

$$c = a \cdot b$$

Desired initial concentration: cc_i

Needed amount of cells for total volume e where

$$e = c \cdot cc_i \text{ (cells)}$$

Concentration of mother culture cc_m

Volume of mother culture needed to acquire e amount of cells V_m where

$$V_m = \frac{e}{cc_m} \text{ (mL)}$$

Volume of 1X Medium E with 5 g/L glucose needed to achieve c, V_e:

$$V_e = c - V_m \quad \text{(mL)}$$

For example, if ten individual cultures want to be started (a = 10) in 10 mL test tubes (b = 10 mL) then 100 mL ($c = 10 \cdot 10\text{mL} = 100\text{mL}$) of total volume will be needed. If the desired initial concentration of these cultures is $cc_i = 1\times10^5\ {}^{\text{cell}}/_{\text{mL}}$ then 1×10^7 cells ($e = c \cdot cc_i = 100\text{ mL} \cdot 1\times10^5\ {}^{\text{cell}}/_{\text{mL}} = 1\times10^7$cells) will be needed. If the concentration on the mother culture was calculated to be $1.01\times10^7\ {}^{\text{cells}}/_{\text{mL}}$ then the volume of mother culture needed to create these cultures is 1.01 mL $\left(V_m = \frac{e}{cc_m} = \frac{1\times10^7\text{cell}}{1.01\times10^7\ {}^{\text{cell}}/_{\text{mL}}} = 0.99\text{ mL}\right)$. Finally, the volume of dilutant (1X Medium E with 5 g/L glucose) can be calculated to be $V_e = c - V_m = 100\text{ mL} - 0.99\text{ mL} = 99.1\text{ mL}$. Grandmother cultures were diluted trying to achieve a $cc_i = 6.75\times10^5\ {}^{\text{cell}}/_{\text{mL}}$ starting concentration.

The required amount of mother culture and of 1X Medium E with 5 g/L glucose were then introduced into a sterile Erlenmeyer where it could be thoroughly stirred prior to distributing it to the individual test tubes. The amount of air was minimized in the test tubes and constant in size along all test tubes. These test tubes are screw-capped and don't allow for air to leak through. The cultures were placed in an incubator at 30°C and the activation time was recorded. The tubes were not disturbed to allow the cells to freely sediment. On an hourly basis, or as required, one test tube was removed from the incubator, shaken and stirred to make the solution homogeneous and a sample placed on the hemocytometer and/or Spectrophotometer. After taking that sample, the rest of the contents of the test tube were discarded. The OD/concentration values were recorded for data analysis.

APPENDIX 2 - AES-1 TIMELINE

Table 19. AES-1 detailed timeline. All dates and times are MDT. Dates are in the mm/dd/yy format. The 24-hour time format is used for time. FPA = Fluid Processing Apparatus. ETC = Environmental Test Chamber. CGBA = Commercial Generic Bioprocessing Apparatus. GAP = Group Activation Pack. ISS = International Space Station. MELFI = Minus Eighty Degrees Laboratory for ISS.

Environment	Event	Date	Time
Earth	Preparation of growth medium	12/3/14	
Earth	Flight-ready hardware parts received	12/3/14	
Earth	Loading of growth medium in FPA Chamber A	12/4/14	
Earth	Start of experiment hardware assembly	12/5/14	
Earth	Preparation of PBS, paraformaldehyde, and RNA solutions	12/5/14	
Earth	Breaking of RNA crystals	12/6/14	
Earth	Confirmation of no-contamination in FPAs Chamber A	12/8/14	
Earth	Culture of AES-1 *E. coli* batch	12/8/14	
Earth	Inocula preparation	12/9/14	
Earth	Loading inocula into FPA Chamber B	12/9/14	
Earth	Preparation of Antibiotic Solutions	12/10/14	
Earth	Loading antibiotic into FPA Chamber C	12/10/14	
Earth	Shipping of all (flight and ground controls) to NASA Wallops	12/10/14	
Earth	Loading fixative to FPA Chamber D	12/12/14	
Earth	GAP assembly	12/13/14	
Earth	GAP pre-flight testing	12/14/14	
Earth	Handover for launch	12/13/14	7:30
Earth	*Planned* Orbital CRS-1 launch	12/17/14	
Earth	*Actual* Orbital CRS-1 launch	9/1/14	11:07
Space	Cygnus berths to ISS	12/1/14	3:55
Space	CGBA transferred from Cygnus to ISS	13/1/14	8:25
Earth	GAPs placed on Clinostat in ETC. ETC starts to ramp up to 30C (similar profile to CGBA2)	13/1/14	13:25
Space	Inoculation	14/1/14	9:40

Space	AB introduction	14/1/14	4:55
Earth	Inoculation	14/1/14	14:40
Earth	AB introduction	15/1/15	9:55
Space	Fix. Introduction	15/1/15	11:05
Space	Insertion in MELFI	15/1/15	11:30
Earth	Fix. Introduction	16/1/14	16:05
Earth	Storage into Freezer	16/1/14	16:30
Earth	SpaceX-3 Splashdown	5/18/14	3:00
Earth	SpaceX-4 Splashdown	10/25/14	13:38

APPENDIX 3 - AES-1 TEMPERATURE PROFILES

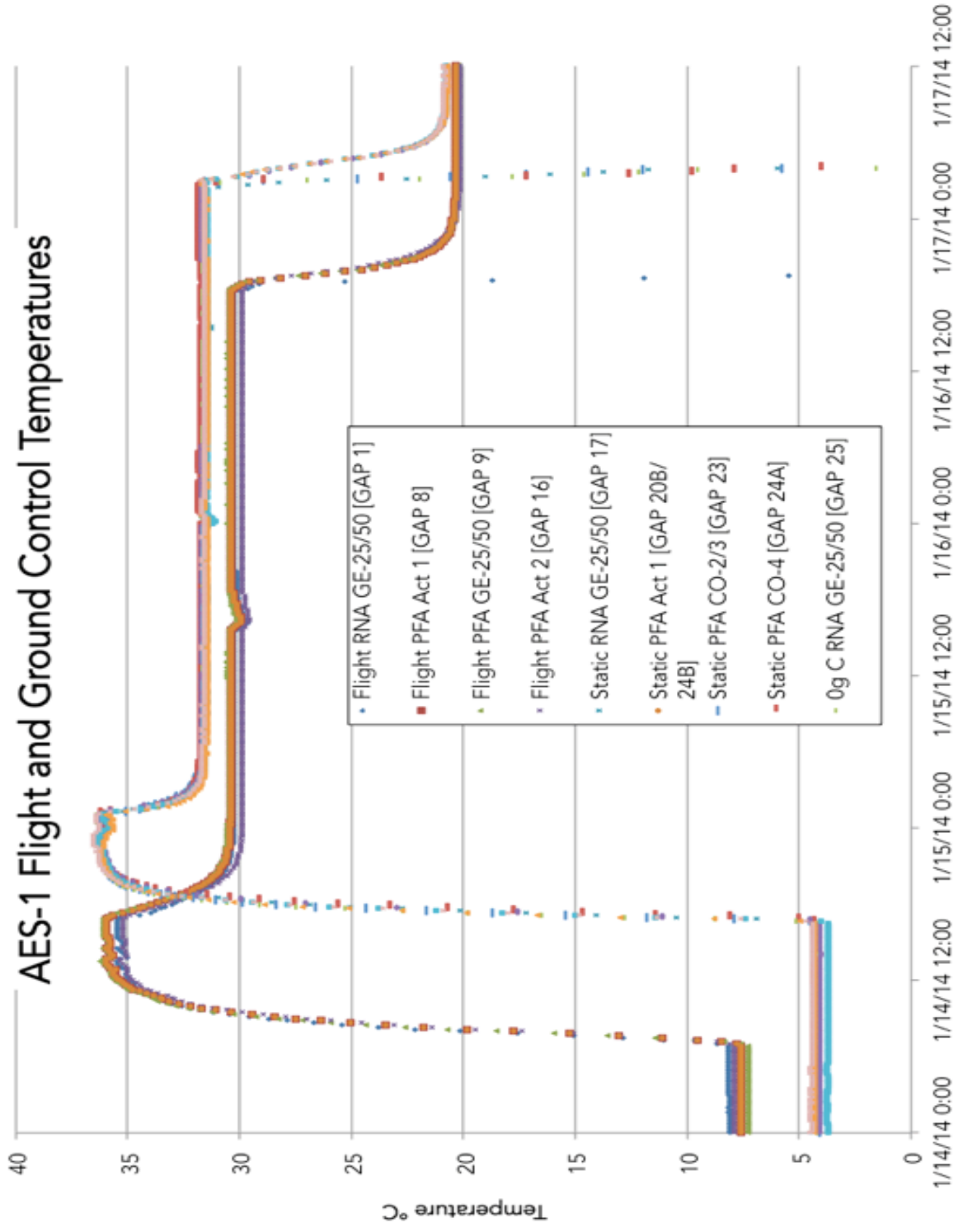

Figure 33. AES-1 flight and ground control temperatures.

APPENDIX 4 - PAPERS PRODUCED FROM THIS WORK

Journal Papers

1. **Zea, L.,** Prasad, N., Levy, S., Larsen, M., Qvortrup, K., Moeller, R., Stodieck, L., Klaus, D., *Phenotypic and Gene Expression Changes in E. coli Challenged with Antibiotics during Spaceflight, (in work)*
2. **Zea, L.,** Stodieck, L., Klaus, D., *Changes in E. coli growth and cell size under simulated reduced levels of gravity*, (*in work*)
3. **Zea, L.,** *Antibiotic Activity and Bacterial Cellular Envelope Investigations in Space, (in work)*
4. **Zea, L.,** *Microbiological Experiments Onboard CubeSats, (in work)*

Conference Papers

5. **Zea, L.,** *Microbiological Experiments Onboard CubeSats – A Review and Prospects*, IAA-BR-14-13-03, 1st Latin American IAA CubeSat Workshop, Brasilia, Brazil, December 8-11, 2014

Conference Abstracts and Posters

6. **Zea, L.,** Stodieck, L. and Klaus, D., *Preliminary Results of the Antibiotic Effectiveness in Space-1 (AES-1) Experiment Conducted Onboard ISS*, ASGSR Conference, Pasadena, CA, October 22-26, 2014
7. **Zea, L.,** Stodieck, L. and Klaus, D., *The First Fifty Years of Bacterial Growth and Antibiotic Effectiveness Research in Space*, ASGSR Conference, Pasadena, CA, October 22-26, 2014
8. **Zea, L.,** Stodieck, L., Klaus, D.M., *Bacterial Growth and Susceptibility to Antibiotics in Simulated Reduced Levels of Gravity*, ASGSR Conference, Orlando, FL November 3-8, 2013

Technical Presentation, Seminars & Workshops

9. **Zea, L.,** *Space Life Sciences and Antibiotic Effectiveness in Space*, HudsonAlpha Seminar, HudsonAlpha, Huntsville, AL, Oct 30, 2014
10. **Zea, L.,** *Space-Based Life Sciences Research and its Benefits to the General Public*, Icelandic Academy of Sciences, University of Reykjavik, Iceland, August 11, 2014 *(invited)*
11. **Zea, L.,** *Bacteria in space: Statistical facts of the first 50 years of research and an experiment currently being conducted onboard ISS*, German Aerospace Center's (DLR) Institute of Aerospace Medicine Seminar, Cologne, Germany, July 22, 2014
12. **Zea, L.,** *Characterization of Gravity's Influence on Mass Transport Phenomena and its Impact on E. Coli's Behavior and Susceptibility to Antibiotics*, BioFrontiers Seminar, University of Colorado, Boulder, February 13, 2013
13. **Zea, L.,** *Spaceflight Bacteria-related Phenomena and their Implications on Astronaut Health*, Gravitational Biology Seminar, German Aerospace Center, Cologne, Germany, May 8, 2014
14. **Zea, L.,** *Some of BioServe's Flown Payloads to Space – From Cells to Mice*, Astrobiology Seminar, Institute of Aerospace Medicine, DLR, Cologne, Germany, May 28, 2014

15. **Zea, L.,** *Student European Low Gravity Research Association,* German Aerospace Center, Cologne, Germany, July 4, 2014

APPENDIX 5 - LIST OF AES-1 DIFFERENTIALLY EXPRESSED GENES

List of differentially expressed genes in the 25 μg/mL samples.

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
aaeA	-2.62	argD	-4.77	bioH	-4.61	crcA_1	2.11	dedA	-3.47	elaB	2.03
aaeR	-2.85	argG	-5.48	bipA	-4.69	creB	-2.08	dedD	-2.43	elaD	4.17
aaeX	-3.05	argH	-2.33	bisC	-2.39	creC	-2.03	def	-2.32	elbB	-2.15
aas	-2.34	argO	-2.03	bolA	2.15	crl	-5.13	degP	-11.53	emrA	-5.27
abgR	-2.92	argP	-3.04	borD_1	-2.41	csdA	-3.84	degQ	-2.02	emrB	-4.81
accA	-2.3	argS	-8.07	borD_2	-2.43	csgC	-3.7	degS	-3.18	emrD	-5.15
accB	-5.5	argT	-2.51	brnQ	-3.23	csgD	2.26	deoR	-2.82	emtA	-2.72
accC	-4.14	arnA	-2.72	bssS	2.84	csgG	-3.09	der	-6.78	entD_1	-2.32
accD	-3.36	aroA	-2.12	btuB	-2.14	csiR	-2.07	dfp	-5.77	entS_1	2.05
ackA	-3.28	aroB	-3.09	btuC	-2.11	cspA	-7.61	dgkA	-2.63	envC	-3.33
acnB	-2.44	aroC	-3.1	btuD	-2.24	cspG	-17.79	dgoR	-2.47	envZ	-4.3
acpT	-3.09	aroG	-3.88	btuF	-2.12	cspH	-2.65	dgoT	-2.17	epd	-2.53
acrA	-2.22	aroK	-4.45	btuR	-2.5	csrA	-3.36	dhaR	-2.95	essD_1	-2.02
acrB	-2.03	arpA	5.05	caiD	-8.99	cstA_1	-4.24	diaA	-5.78	essD_2	-2.28
acrR	-2.11	artM	-2.11	caiE	-6.92	cstA_2	-4.59	dinB	-2.37	eutA	-2.17
add	-4.91	artP	-2.94	caiF	-3.03	cusB_1	-2.32	dinD	-6.94	eutB	-6.07
adhE	2.61	artQ	-2.05	cbpA	2.55	cusF_1	-2.13	dinF	-2.59	eutH	-2.05
adiC	2.91	ascB	-2.4	cbpM	3.41	cusF_2	-3.94	dinG	-3.64	exoX	-3.94
adk	-6.48	ascF	-2.49	cca	-5.59	cusR_1	-2.78	dinI	-7.78	exuR	-4.06
aegA	-4.51	ascG	-2.92	cchB	-3.32	cusR_2	-2.42	djlA	-4.37	fabA	-5.85
aer	-3.34	aslB	-2.66	ccmA	-2.89	cutC	-2.26	djlB_1	-2.18	fabB	-3.19
agaA	-2.26	asmA	-2.2	cdaR	-4.52	cvpA	-3.34	djlB_2	-3.04	fabD	-3.24
agaR	-3.28	asnC	-2.88	cdd	-2.32	cvrA	-2.43	dkgA	2.91	fabF	-3.61
agaV	-3.71	asnS	-2.23	cdh	-2.01	cybB	-4.04	dksA	-2.38	fabG	-2.48
agp	2.92	aspC	-2.87	cdsA	-3.42	cycA	-2.05	dmsA	-2.96	fabH	-4.52
aidB	6.41	aspS	-2.57	cedA	-5.12	cydD	-3.67	dmsB	-2.31	fabI	-2.83
alaS	-6.13	atpA	-2.38	chaA	-7.81	cyoA	-3.34	dmsD	-2.15	fabR	-4.27
allA	-2.03	atpB	-4.06	chaC	-3.76	cysB	-2.25	dnaB	-5.1	fabZ	-3.18
allB	-2.05	atpE	-3.45	chbB	-2.13	cysC	-2.4	dnaE	-2.28	fadA	-2.11
allR	3.75	atpF	-2.73	cho	-2.08	cysM	-2.5	dnaG	-2.44	fadB	-2.02
alr	-4	atpG	-2.29	chpA	-3.15	cysS	-7.94	dnaJ	3.02	fadD	-5.21
alsE	-2.32	atpH	-2.69	chpR	-6.57	cysU	-2.27	dnaK	7.57	fadE	-2.79
alsK	-2.97	atpI	-4.12	clcA	-2.14	cysZ	-10.61	dnaT	-2.05	fadH	-2.4
alx	-5.02	avtA	-2.65	clcB	-2.53	cytR	-5.27	dnaX	-3.69	fadR	-5.49
amiA	-2.25	bacA	-4.24	cld	-7.38	dacA_1	-3.44	dps	4.78	fbaA	2.91
amiB	-2.88	baeR	-2.42	clpB	8.76	dacA_2	-3.33	dsbA	-2.16	fbaB	3.21
amiC	-4.8	bax	-8.72	clpX	-2.29	dacB	-3.68	dsbB	-3.51	fbp	-4
ampG	-3.7	bcp	-3.75	cls	-2.61	dadA	-3.31	dsbC	-2.44	fdhD	-2.97
anmK	-3.2	bcr	-3.46	cmk	-10.06	dadX	-3.43	dtd	-2.56	fdhE	-3.38
ansA	-2.56	bcsA	-3.99	cmr	-4.63	dam	-2.71	dusA	-4.45	fdhF	-5.16
ansB	-2.88	bcsF	-2.17	coaA	-2.14	dapA	-4.79	dusB	-11.99	fdnG	-4.35
aphA	-2.19	bdm	-4.8	coaE	-2.45	dapD	-2.51	dusC	-4.41	fdnH	-4.54
appA	9.46	betB	-2.95	cobB	-2.18	dapE	-3.56	dut	-5.84	fdnI	-4.17
appB	7.66	betI	-3.69	cobS	-3.6	dapF	-2.81	dxs	-3.43	fdoG	-3.28
appC	4.96	betT	-2.73	cobT	-2.83	dbpA	-2.45	eamA	-2.16	fdoH	-5.24
apt	-12.5	bfd	2.88	cobU	-4.1	dcd	-3.61	ebgC	2.35	fdoI	-4.2
aqpZ	-2.67	bfr	3.8	cof	-2.8	dcm	-2.52	ecfK	-6.74	feaB	-2.52
araF	-2.41	bglF	-2.87	corA	-3.57	dcuS	-2.17	ecnA	-34.31	fecI	-2.02
arcA	-2.58	bglG	2.56	cpdA	-2.77	ddlB	-3.09	ecnB	15.7	fes_2	2.18
argA	-4.64	bglH	-2.37	cpxA	-2.88	ddpD	-2.86	eco	-3.47	ffh	-2.35
argB	-2.21	bglJ	2.13	cpxP	-8.58	ddpF	-5.35	efeO	2.02	fhuE	-2.07
argC	-3.05	bglX	-3.54	cpxR	-2.44	deaD	-6.23	efp	-9.34	fieF	-2.48

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
fimZ	2.53	gabT	3.39	gpp	-3.17	hrpB	-3.6	insG	-4.05	lpxC	-7.14
fis	-6.61	gadA	7.82	gpt	-8.3	hslJ	-4.31	insL-1	-2.01	lpxD	-2.9
fklB	-5.16	gadB	5.75	greA	-6.76	hslO	4.65	insL-2_2	-2.1	lpxK	-4.9
fkpB	-5.66	gadC	8.96	greB	-2.55	hslR	8.4	insL-3	-2.6	lpxL	-4.57
fldA	-2.62	gadE	20.62	groL	5.22	hslU	2.35	insO-1	-2.67	lpxM	-3
fldB	-3.02	gadW	6.04	groS	4.62	hslV	2.63	insO-2	-2.74	lpxP	-3.38
flgM	-4.84	gadX	7.21	grpE	3.7	htpG	5.86	ispA	-7.66	lrhA	-2.62
flgN	-3.81	galE	-2.44	grxC	-2.11	htrC	-3.05	ispB	-2.97	lrp	-2.42
flhE	-3.21	galK	-2.86	grxD	-2.27	htrG	-8.27	ispD	-2.37	lspA	-9.77
fliA	-2.08	galM	-3.3	gshB	-2.65	hupA	-2.33	ispE	-2.78	lsrK	-4.19
fliE	-3.46	galR	-2.46	gsiA	-2.31	hupB	-2.3	ispG	-2.87	lysA	-4.12
fliG	-2.07	galT	-2.19	gsk	-3.32	hyaA	6.52	ispH	-4.27	lysP	-3.19
fliN	-2.08	galU	-3.59	guaA	-3.27	hyaB	8.37	ispU	-3	lysR	-2.17
fliO	-2.01	garD	-5.6	guaB	-4.99	hyaC	13.65	ivbL	-3.15	lysS	-2.57
fliR	-4.67	gatA	2.83	gyrA	-3.75	hyaD	12.2	ivy	-8.76	maa	-2.75
flk	-2.18	gcd	-3.11	hcaE	-2.53	hyaE	6.9	kbl	-2.42	macB	2.99
fmt	-2.92	gdhA	-2.17	hcaT	-3	hyaF	8.03	kdgK	-4.37	maeB	-3.16
fnr	-2.41	gfcD	-2.2	hchA	2.17	hybO	-2.38	kdgR	-2.14	mak	-2.14
focA	-2.47	gidA	-2.53	hda	-3.93	hycA	-6.76	kdpA	-4.43	malE	2.81
focB	-2.07	glcC	-3.8	hdeA	4.49	hycB	-5.79	kdpB	-2.82	malM	-2.05
folA	-2.84	glgS	2.81	hdeB	4.78	hycC	-3.72	kdpD	-2.34	malP	-2.01
folB	-2.82	glmM	-2.34	helD	-2	hydN	-4.18	kdsB	-3.4	malQ	-6.38
folD	-2.83	glmU	-2.33	hemA	-3.95	hyi	2.4	kdsC	-2.51	malT	-2.17
folE	-2.55	glnB	-2.04	hemB	-2.25	hypA	-3.4	kdsD	-2.85	manA	-5.89
folK	-2.1	glnG	-2.17	hemC	-3.59	hypB	-2.26	kefA	-3.67	map	-2.44
folM	-2.9	glnP	-2.26	hemD	-2.83	hypC	-2.63	kefB	-2.39	marA	-3.25
folP	-2.1	glnQ	-2.09	hemE	-2.67	hypD	-2.19	kgtP	-3.68	marB	-3.23
folX	-3.07	gloA	-2.44	hemF	-2.31	hypF	-2.04	ldcA	-3.11	marR	-5.06
fre	-2.04	glpA	-7.47	hemG	-2.71	iaaA	-2.43	ldrB	-2.7	mazG	-2.67
frlR	-2.5	glpB	-2.99	hemL	-2.97	iap	-3.97	leuS_1	-5.5	mdlA	-3.25
frmA	-5.96	glpD	-3.32	hemY	-2.84	ibpA	6.81	leuS_2	-5.41	mdlB	-2.88
frmB	-3.95	glpE	-7.19	hflD	-3.17	ibpB	6.68	lexA	-3.12	mdoC	-2.27
frmR	-6.38	glpF	-2.64	hfq	2.55	icd	-3.08	lgt	-2.28	mdoD	-2.73
frvB	-3.01	glpG	-4.61	hha	-3.14	ileS	-3.18	lhr	-2.25	mdoH	-2.25
frvX	-2.07	glpK	-2.42	hinT	-3.24	ilvA	-3.08	ligA	-2.08	mdtA	-4.25
fsaA	-2.43	glpR	-4.07	hipB	-2.08	ilvB	-3.13	ligT	-2.87	mdtE	5.68
fsr	-2.53	glpT	-2.38	hisB	-2.06	ilvE	-4.54	livH	-3.75	mdtF	3.37
ftn	-4.38	glpX	-3.38	hisC	-2.05	ilvM	-7.07	livJ	-2	mdtG	-7.79
ftsA	-5.32	gltA	2.35	hisD	-3.08	ilvN	-2.02	livK	-4.59	mdtH	-2.5
ftsB	-2.79	gltF	-2.17	hisG	-4.65	ilvY	-2.64	livM	-2.77	mdtJ	-4.43
ftsE	-3.9	gltP	-2.76	hisJ	-3.28	imp	-5.45	lldP	-2.18	mdtK	-2.44
ftsH	-3.36	gltX	-2.69	hisQ	-3.36	inaA	-2.07	lolA	-4.18	menA	-2.75
ftsI	-2.46	glyA	-2.03	hisS	-3.72	infA	-3.7	lolB	-2.76	menB	-2.08
ftsL	-2.93	gmhB	-2.45	hlyE	13.12	infB	-3.28	lolC	-2.96	menC	-2.04
ftsN	-5.46	gnsA	-5.29	hns	-4.52	insA-2	-2.84	lolD	-2.3	menD	-2.3
ftsQ	-3.81	gntK	-4.33	hokA	-4.61	insA-4	-6.28	lomR	-2.43	menF	-2.01
ftsW	-2.8	gntR	-4.45	holB	-3.92	insA-6	-2.74	lon	2.52	mepA	-2.23
ftsX	-3.83	gntT	-2.53	holD	-4.34	insA-7	-2.63	lpcA	-3.08	metB	-2.08
ftsY	-4.65	gntU	-3.34	holE	-5.72	insB-1	-2.44	lplA	-2.24	metC	-4.23
ftsZ	-6.64	gntX	-4.13	hpf	-2.51	insB-4	-2.94	lpp	3.73	metG	-2.51
fur	-2.49	gph	-2.05	hpt	-4.71	insE-3	3.37	lpxA	-4.15	metJ	-2.18
fxsA	3.9	gpmI	-5.05	hrpA	-2.74	insE-5	2.3	lpxB	-3.55	metL	-2.03

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
mfd	3.46	mutH	-2.17	nusA	-4.95	pldA	-8.27	ptsN	-2.25	rhaR	-2.59
mgsA	-2.33	mutL	-2.76	nusG	-3.95	plsB	-2.68	purE	-2.19	rhaS	-2.2
mgtA	-5.05	mutM	3.89	obgE	-7.49	plsX	-5.5	purK	-2.07	rhlB	-4.39
miaA	2.69	mutS	-3.55	ogt	-2.27	pmrD	-5.88	purM	-3.92	rhlE	-6.16
miaB	-3.07	mutY	-3.79	ompC	-2.21	pncA	-2.07	purN	-3.81	rho	-2.32
minC	-3.67	nadA	-3.8	ompR	-3.55	pncB	-3.33	purT	-2.05	rhoL	-3.31
minD	-2.96	nadB	-5.64	ompT_1	-2.04	pnp	-5.21	purU	-2.7	rhsB	-3.23
mioC	-4.87	nadC	-3.42	ompT_2	-2.03	pntA	-2.6	putP	-2.52	rhsC	-4.22
mipA	-7.37	nadE	-2.06	ompW	-2.91	pntB	-2.11	puuA	-13.61	rhtA	-3.87
mltA	-5.78	nagD	-5.29	oppB	2.67	pnuC	-3.63	puuB	-2.62	ribB	-3.99
mltB	-3.08	nagE	-2.88	oppC	3	potA	-6.99	puuC	-5.4	ribD	-2.06
mltC	-2.32	nanR	-2.38	oppD	4.36	potB	-4.11	puuD	-7.2	ribF	-7
mltD	-5.04	napB	-2.27	oppF	4.08	potE	-2.47	puuE	-2.27	rihC	-3.49
mnmA	-3.93	napC	-3.17	orn	-2.68	poxA	-7.85	puuP	-4.63	riml	-3.02
mntH	-2.13	napD	-7.65	osmB	-8.74	poxB	4.7	puuR	-9.57	rimM	-10.3
mntR	-3.41	napF	-7.52	osmC	-4.55	ppa	-4.05	pyrE	-2.66	rlmL	-2.55
moaA	-2.96	napH	-2.28	oxyR	-3.41	ppdB	-2.13	pyrH	-2.38	rlpB_1	-4.42
moaC	-2.02	narH	2.82	paaJ	-2.85	ppdD	-2.02	pyrL	-6.17	rlpB_2	-4.03
moaD	-2.27	narI	2.47	pabA	-2.87	ppiA	-4.38	qmcA	-2.12	rluA	2.06
modA	-2.33	narJ	2.67	pabC	-3.36	ppiC	-4.57	qseB	-2.25	rluB	-5.18
modB	-3.02	narP	-2.16	pal	-2.93	ppiD	-4.63	queA	-4.28	rluC	-5.22
modC	-3.18	ndk	-7.35	panB	-3.6	pps	-4.05	queC	-3.54	rluE	-6.48
modE	-3.55	nemA	-3.55	panF	-2.52	pptA	-2.5	queF	-2.44	rluF	-2.97
moeA	-3.04	nfnB_1	-2.55	parC	-2.86	pqiA	-3.35	radA	-3.18	rmf	2.29
moeB	-2.85	nfnB_2	-2.66	pcnB	-8.94	pqiB	-2.34	rarD	-4.02	rmuC	-3.27
mokC	2.27	nfo	-3	pdhR	-4.39	prc	-2.2	rbfA	-2.82	rna_1	-2.51
motA	-2.97	nfrB_1	-2.45	pdxA	-3.91	prfA	-2.26	rcnR	-2.85	rna_2	-2.54
motB	-2.91	nfrB_2	-2.06	pdxB	-2.65	prfB	-2.99	rcsA	-4.12	rnb	-2.51
mpaA	-2.25	nfsA	-2.5	pdxH	-2.43	prfC	-2.64	rcsB	-3.78	rnd	-3.07
mpl	-3.13	nhaB	-3.62	pdxY	-2.28	priA	-2.71	rcsC	-3.92	rnhA	-2.81
mqo	-2.31	nhaR	3.36	pepA	-3.56	priC	-2.25	rcsD	-6.76	rnhB	-3.34
mraW	-3.97	nikA	-2.95	pepE	-2.75	prmB	-3.45	rcsF	-2.78	rnk_1	-6.76
mraZ	-6.1	nirD	2.7	pepQ	-2.18	prmC	-2.22	rdgC	-2.24	rnk_2	-6.6
mrcA	-5.42	nlpB	-6.11	pepT	-2.04	proA	-3.12	recB	-2.45	rnpA	-24.83
mrcB	-6.22	nlpC	-2.32	pfkA	-2.41	proB	-3.1	recC	-5.61	rnt	-6.58
mrdA_1	-3.5	nlpD	-4.5	pfs	-2.43	proC	-2.78	recD	-3.77	rob	-2.97
mrdA_2	-2.63	nlpE	-2.93	pgaC	-3.65	proP	-5.15	recJ	-3.88	rof	-2.07
mrdB_1	-2.42	nlpI	-2.34	pgm	-2.57	proQ	-3.77	recN	-3.84	rpe	-2.11
mreB	-3.9	norR	-3.44	pgsA	-2.35	proS	-5.57	recQ	-4.73	rpiA	-2.76
mreD	-2.3	norV	-2.12	pheP_1	-3.21	proW	-2.01	recR	-4.36	rplA	-7.25
mrp	-2.29	nrdA	-2.07	pheP_2	-3.96	proY	-4.17	recX	-12.46	rplB	-9.88
msbA	-7.57	nrdH	-3.53	pheS	-2.2	prsA	-3.4	relA	-2.35	rplC	-11.36
mscL	2.19	nrdI	-2.32	phnA	-12.95	psd	-2.95	rep	-2.61	rplD	-9.94
mtlA	2.15	nrdR	-2.4	phnG	2.44	psiF	3.77	rfaB	-2.72	rplE	-7.26
mtlD	3.25	nrfA	-2.11	phnL	-2.26	pspA	-7.03	rfaE	-2.21	rplF	-6.24
mukB	-2.65	nudB	-3.25	phnO	-2.84	pspB	-7.83	rfaG	-3.8	rplI	-5.81
mukE	-4.62	nudE	-5.28	phnP	-6.58	pspC	-7.78	rfaH	-2.99	rplJ	-9.56
mukF	-3.11	nudF	-2.17	phoB	-3.3	pspD	-7.29	rfaP	-3.02	rplK	-6.92
murC	-2.66	nudG	-2.57	phoR	-3.56	pspE	-3.82	rfaQ	-2.97	rplL	-7.04
murF	-2.06	nudJ	-2.94	phoU	2.56	pssA	-2.48	rfaZ	-2.68	rplM	-10.34
murG	-3.77	nuoA	-2.14	php	-2.34	pth	-3.36	rfe	-3.12	rplN	-6.97
murP	-2.51	nuoB	-2.64	pitA	-2.81	ptrA	-3.44	rhaB	2.09	rplO	-6.19

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
rplP	-6.22	rspA	-2.23	srmB	-2.91	torA	-2.18	uup	-2.66	ybaW	-2.61
rplQ	-4.16	rspB	-2.44	ssb	-4.43	torR	-3.69	uvrC	-2.55	ybaZ	-3.82
rplR	-5.64	rstA	-2.58	sscR	-7.23	torS	-2.67	uvrD	-6.23	ybbA	-2.67
rplS	-8.24	rsuA	-7.22	sspA	-2.81	torY	-3.49	uvrY	-4.36	ybbB	-2.69
rplU	-8.11	rsxA	-4.17	sspB	-3.08	torZ	-3.44	vacJ	-3.38	ybbJ	-2.07
rplV	-9.05	rsxB	-3.01	sthA	-2.32	tppB	-2.26	valS	-2.08	ybbM	2.07
rplW	-11.25	rtcR	-2.06	stpA	-2.54	tpr	-2.3	wcaJ	-2.14	ybcI	-4.78
rplX	-6.07	rumA	-2.73	sucA	2.7	tpx	-2.59	wcaK	-2.39	ybcJ	-2.35
rplY	-4.54	rumB	-2.57	sucB	3.91	tqsA	-3.63	wrbA	22.71	ybdD_1	-2.07
rpmA	-7.48	rutE	-2.58	sucC	3.33	treB	-2.74	xapR	-2.34	ybdG_1	-4.44
rpmB	-5.76	rutR	-2.34	sucD	4.22	treR	-3.25	xerD	-3.12	ybdG_2	-4.6
rpmC	-5.84	ruvA	-3.03	sufA	4.99	trkH	-2.07	xseA	-3.25	ybdJ_1	-3.83
rpmD	-7.05	ruvB	-2.13	sufB	2.86	trmA	-4.62	xseB	-3.47	ybdJ_2	-5.87
rpmE	-2.49	ruvC	-3.83	sufC	2.42	trmD	-9.75	xthA	-4.25	ybeA_1	-5.17
rpmF	-8.14	rzpR	-2.15	suhB	-5.12	trmI	-4.89	yaaA	-2.23	ybeA_2	-5.98
rpmG	-2.76	sanA	-2.07	sulA	-4.69	trpH	-2.83	yaaX	-12.95	ybeB_1	-7.94
rpmH	-12.11	sbcB	-2.7	surA	-10.86	trpR	-5.12	yacC	-3.45	ybeB_2	-5.92
rpmJ	-3.52	sbmA	-2.79	syd	-2.69	trpS	-2.36	yacF	-2.18	ybeD_1	2.08
rpoA	-4.95	sdhC	-2.07	tadA	-4.75	truA	-5.3	yadB	-2.44	ybeD_2	2.12
rpoB	-2.06	secA	2.65	talA	2.55	truB	-2.95	yadG	-2.04	ybeF_2	-2.36
rpoE	-2.67	secB	-2.45	tas	-2.55	truC	-4.2	yadI	-2.19	ybeY	3.5
rpoN	-2.77	secD	-4.87	tatA	-3.15	trxB	-3.06	yaeB	-2.22	ybeZ	3.26
rpoS	-3.48	secE	-5.23	tatB	-3.11	tsf	-2.58	yaeJ	-2.4	ybfB	-2.23
rpoZ	-2.03	secF	-4.01	tatC	-3.11	tsgA	-3	yaeQ	-2.43	ybfD	-2.58
rpsA	-3.53	secG	-4.64	tatD	-2.56	tsx	-4.56	yaeR	-6.03	ybfE	-3.62
rpsB	-3.44	secM	-2.7	tauB	2.06	ttk	-3.35	yafJ	-2.89	ybfF	-3
rpsD	-3.97	secY	-6.29	tdcA	2.23	tufB	-4.52	yafN	-2.4	ybfN	-2.48
rpsE	-6.92	selD	-3.41	tdcB	2.15	tyrP	-2.85	yafV	-2.19	ybfO	-3.12
rpsF	-10.68	seqA	-2.09	tdcD	2.05	tyrS	-4.3	yagU	-2.42	ybfP	-9.74
rpsH	-7.1	serB	-4.35	tesA	-2.54	ubiB	-2.28	yahA	-5.02	ybfQ	-3.92
rpsI	-10	setB	-3.95	tesB	-3.66	ubiE	-4.46	yahB	-2.05	ybgC	-3.21
rpsJ	-12.5	sfsB	-2.44	tgt	-4.27	ubiF	-2.53	yaiE	-2.89	ybgD	-2.08
rpsK	-4.27	sgbE	-2.3	thiE	2.84	ubiG	-5.45	yaiL	-2.86	ybgF	-2.08
rpsM	-3.71	sgbH	3.01	thiF	3	ubiX	-3.37	yaiO	-2.14	ybgH	-2.99
rpsN	-6.99	sirA	-6.36	thiG	2.34	ucpA	-2.86	yaiT	-2.83	ybgI	-2.66
rpsO	-2.79	slp	9.84	thiH	2.37	udk	-2.23	yaiW	-4.69	ybgJ	-2.9
rpsP	-9.62	slt	-2.23	thiI	-2.95	udp	-2.22	yaiY	-11.56	ybgS	2.27
rpsQ	-4.57	slyA	-2.78	thiK	-2.13	ugd	-2.57	yaiZ	-3.02	ybhA	-3.48
rpsR	-9.33	slyB	-2.56	thiS	3	ugpE	2.77	yajC	-2.32	ybhC	-2.46
rpsS	-10.25	slyD	-2.64	thrA	-6.28	uhpA	-5.66	yajD	-4.18	ybhF	-2.53
rpsT	-12.33	smpA	-2.74	thrB	-3.07	uhpB	-5.2	yajG	-7.08	ybhJ	-2.14
rpsU	-6.51	smpB	-2.39	thrS	-2.03	ulaC	-2.37	yajI	-12.05	ybhK	-2.18
rraA	-2.23	smtA	-3.56	thyA	-2.02	ulaD	-2.13	yajQ	-3.14	ybhO	2.28
rrmA	-5.61	speB	-3.73	tiaE	-2.79	ulaE	-5.1	yajR	-3.13	ybhQ	-5.3
rrmJ	-3.11	speC	-2.2	tig	-4.28	ulaF	-4	ybaB	-3.64	ybhR	-3.01
rsd	-3.99	speE	-4.12	tilS	-4.05	ulaR	-2.28	ybaJ	-2.02	ybhS	-2.29
rseA	-3.06	speG	-2.65	tmk	-5.9	umuD	-3.99	ybaM	-2.56	ybiH	-4.16
rseB	-2.12	spr	-4.27	tolA	-3.02	ung	-2.64	ybaO	-3.63	ybiM	3.83
rseP	-6.8	sprT	-4.61	tolB	-3.44	upp	-5.67	ybaQ	-2.34	ybiN	-5.08
rsgA	-3.73	sra	7.09	tolQ	-6.01	usg	-3.05	ybaS	6.95	ybiP	-2.3
rsmB	-2.01	srlA	-2.14	tolR	-3.21	uspB	2.51	ybaT	3.74	ybiR	-3.97
rsmC	-2.46	srlD	-2.22	topB	-5.14	uspE	2.02	ybaV	-2.86	ybiS	-9.62

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
ybiT	-6.24	ycgJ	-2.32	ydgH	-2.77	yedA	-2.41	yfbV	-2.76	yfjG	-2.85
ybiV	-2.74	ycgL	-2.72	ydgI	-2.37	yedD	-3.71	yfcC	-8.89	yfjX	-6.67
ybjC	-2.34	ycgM	-4.1	ydgT	-2.51	yedE	-3.57	yfcD	-2.16	ygaC	-2.82
ybjD	-2.37	ycgN	-2.61	ydhA	-11.36	yedF	-3.74	yfcE	-2	ygaD	-2.24
ybjE	-2.96	ycgR	-2.3	ydhB	-4.22	yedI	-3.93	yfcH	-2.96	ygaF	3.09
ybjG	-5.14	ychE	-2.86	ydhC	-2.41	yedJ	-2.25	yfcI	-2.58	ygaH	-2.63
ybjH	-3.08	ychJ	-2.99	ydhJ	-2.22	yedK	-2.28	yfcJ	-3.27	ygaP	-2.04
ybjJ	-3.28	ychN	-3.57	ydhM	-6.4	yedR	-2.57	yfcL	-9.44	ygaT	2.84
ybjK	-3.6	ychO	-2.25	ydhO	-2.69	yedW	-6.76	yfcM	-11	ygaV	-2.91
ybjN	-3.37	ychQ	-3.24	ydhP	-5	yedX	-2.66	yfcN	-2.74	ygaZ	-2.92
ybjO	-3.45	ychS	-4.63	ydhQ	3.5	yeeD	-3.17	yfdC	-2.06	ygbK	-2.12
ybjP	-2.04	yciA	-2.42	ydhY	-4.71	yeeE	-3.23	yfdX	-3.21	ygbN	-2.27
ybjQ	-4.27	yciC	-2.5	ydiA	-2.17	yeeF	-5.81	yfdY	-2.11	ygbT	-2.03
ybjR	-3.63	yciF	2.91	ydiL	-2.63	yeeS	-2.46	yfeC	-2.97	ygcJ	-2.61
ybjS	-2.66	yciH	-3.76	ydiY	-2.13	yeeT	-2.36	yfeD	-2.55	ygcW	2.44
ybjX	-9.14	yciI	-3.77	ydiZ	2.42	yeeV	-2.16	yfeG	-2.11	ygdB	-2.5
ycaC	6.37	yciK	-3.41	ydjA	-3.36	yeeW	-3.84	yfeK	-6.44	ygdG	-2.95
ycaD	-3.79	yciN	-2.02	ydjM	-8.91	yeeZ	-2.05	yfeR	-3.02	ygdK	-3.25
ycaJ	-3.94	yciT	-4.06	ydjN	-3.53	yefM	-2.17	yfeS	-4.43	ygdL	-2.35
ycaO	-2.5	yciX	-2.94	ydjR	-4.12	yegL	-2.64	yfeU	-3.51	ygdQ	-6.24
ycaQ	-2.42	ycjD	-2.82	yeaG	3.44	yegP	17.92	yfeW	-4.55	ygdR	-10.55
ycaR	-3.99	ycjF	4.86	yeaH	2.5	yegQ	-2.52	yfeX	-5.51	ygeA	-2.47
ycbC	-3.21	ycjG	-2.43	yeaK	-2.63	yegS	-3.94	yfeY	-9.42	ygeD	-3.47
ycbF	-2.27	ycjU	2.21	yeaL	-4.29	yegW	-2.03	yfeZ	-4.78	ygeF	3.08
ycbJ	-2.13	ycjX	6.58	yeaM	-3.31	yehD	-2.01	yffB	-4.31	ygeG	5.17
ycbK	-2.97	ydaL	-2.36	yeaN	-2.81	yehE	3.06	yffH	-2.71	ygeK	2.7
ycbL	-2.75	ydbA	-3.26	yeaP	-3.16	yehL	-3.58	yfgA	-3.53	ygeR	-3.15
ycbW	-7.39	ydbD	-2.28	yeaQ	7.04	yehW	-2.7	yfgB	-3.16	ygfB	-2.2
ycbX	-4.41	ydbH	2.1	yeaS	-4.4	yeiB	-2.23	yfgC	-2.25	ygfG	-2.09
ycbZ	-5.16	ydbJ	-2.83	yeaZ	-5.97	yeiE	-3.46	yfgD	-2.4	ygfK	-3.07
yccA	-4.48	ydcF	-5.19	yebA	-3.15	yeiH	-4.13	yfgG	-2.72	ygfX	-3.32
yccE	2.31	ydcH	2.96	yebC	-3.05	yeiI	-2.43	yfgJ	-5.91	ygfY	-2.89
yccF	-3.71	ydcI	-2.83	yebE	-2.6	yeiL	2.72	yfgL	-9.34	ygfZ	-2.18
yccJ	26.63	ydcM	-2.14	yebF	-8.64	yeiM	-2.32	yfgM	-6.14	yggD	-2.82
yccK	-2.57	ydcP	-3.54	yebG	-7.42	yeiP	-2.64	yfgO	-2.12	yggE	-3.59
yccM	-2.28	ydcS	-2.65	yebN	-6.29	yeiW	-4.19	yfhB	-3.46	yggG	-7.59
yccW	-5.64	ydcX	-2.26	yebO	-2.02	yejG	-2.09	yfhD	-3.1	yggJ	-4.03
ycdX	-2.51	ydcZ	-2.59	yebR	-2.5	yejL	-6.79	yfhK	-2.36	yggL	-2.9
ycdY	-3.22	yddE	-2.01	yebS	-2.78	yejM	-3.66	yfhL	-4.14	yggN	-3.86
ycdZ	-6.14	yddG	-2.09	yebT	-2.1	yfaD	-2.48	yfhQ	-2.61	yggS	-2.52
yceB	-5.61	yddJ	2.38	yebU	-2.28	yfaE	-2.44	yfhR	-3.09	yggT	-2.43
yceD	-13.51	yddM	-10.92	yebZ	-5.34	yfaO	-2.32	yfiA	2.31	yggX	-2.03
yceF	-2.1	yddW	-2.19	yecD	-3.57	yfaU	-2.28	yfiB	-6.56	yghA	2.32
yceG	-6.59	ydeA	-2.64	yecE	-2.87	yfaY	-2.36	yfiC	-3.13	yghB	-2.25
yceN	-2.55	ydeH	-3.26	yecF	-2.51	yfbB	-2.12	yfiE	-2.43	yghD	-6.33
yceQ	-3.97	ydeJ	-4.42	yecH	-5.31	yfbH	-2.61	yfiF	-2.84	yghO	-2.64
ycfJ	-8.18	ydeN	-2.9	yecJ	-2.88	yfbJ	-2.29	yfiK	-3.2	yghW	-2.43
ycfL	-2.31	ydfH	-5.29	yecM	-2.63	yfbN	2.68	yfiN	-2.42	yghZ	-2.84
ycfM	-2.2	ydfI	-3.32	yecO	-2.16	yfbQ	-2.26	yfiO	-3	ygiA	-2.91
ycfR	-2.2	ydfR	2.35	yecP	-2.05	yfbR	-4.34	yfiP	-2.06	ygiB	-3.31
ycfS	-2.02	ydfZ	-2.91	yecR	-2.62	yfbT	-2.83	yfiR	-4.2	ygiC	-2.57
ycgB	3.35	ydgC	-3.24	yecT	-2.02	yfbU	-2.77	yfjF	-2.44	ygiF	-2.4

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
ygiH	-2.27	yhhA	4.35	yihG	-2.02	yjgP	-6.8	yobA	-3.65	ytfB	-2.59
ygiL	2.26	yhhF	-4	yihM	2.36	yjgQ	-6.44	yobB	-3.38	ytfG	-2.85
ygiQ	-4.34	yhhI	-2.01	yihP	-3.23	yjgW	-3.82	yobG	-2.43	ytfH	-2.38
ygiT	-2.03	yhhJ	-2.83	yihW	-2.55	yjhX	-4.67	yobH	-7.39	ytfJ	-2.17
ygiW	3.87	yhhK	-3.05	yiiD	-2.22	yjjB	-2.58	yodC	-2.58	ytfK	4.84
ygiZ	3.59	yhhL	-3.91	yiiF	-2.24	yjjP	-2.11	yoeB	-2.62	ytfN	-2.69
ygjD	-3.3	yhhM	-3.03	yiiL	-7.24	yjjQ	2.81	yohC	9.52	ytfP	-3.61
ygjG	3.76	yhhN	-2.53	yiiM	-2.37	yjjU	-2.39	yohJ	-2.84	ytjB	-2.69
ygjH	2.4	yhhQ	-3.66	yiiT	3.06	yjjV	-2.07	yohK	-2.21	ytjC	-5.33
ygjM	-8.42	yhhS	-4.33	yiiU	-3.19	yjjX	-3.1	yohM	-2.33	zipA	-2.23
ygjN	-13.84	yhiD	3.61	yiiX	-4.69	yjjY	-2.88	yohN	-8.44	zitB	-2.39
ygjO	-4.63	yhiF	4.1	yijD	-5.89	yjtD	-3.25	yohO	-3.5	zntA	-9.61
ygjP	-2.97	yhiJ	2.31	yijE	-3.29	ykgC	2.55	yojL	-2.42	znuA	-2.63
yhaC	2.74	yhiM	6.89	yijO	-2.24	ykgE	-3.01	ypeA	-5.34	znuB	-3.3
yhaH	3.29	yhiN	-2.52	yijP	-2.29	ykgL	-4.18	ypeC	-17.01	znuC	-2.75
yhaI	2.18	yhiR	-2.52	yjaA	-3.71	ykgM	2.83	ypfG	-15.42	zraP	-2.56
yhaJ	-3.12	yhjE	-5.64	yjaG	-4.57	ykiA	-2.98	ypfJ	-2.16	zupT	-2.18
yhaK	-2.03	yhjH	-2.45	yjaH	-6.03	ylaC	-2.38	yqaA	-2.81		
yhaL	-6.24	yhjJ	-8.18	yjbE	2.95	ylbG	-2.21	yqaB	-3.08		
yhaM	-2.61	yhjR	-2.17	yjbF	-2.22	yliG	-7.66	yqcA	-2.54		
yhbC	-5.61	yiaD	-13.51	yjbJ	3.03	ymcE	2.03	yqcC	-9.18		
yhbE	-12.77	yiaG	11.34	yjbL	2.14	ymdC	-2.45	yqeC	-2.43		
yhbG	-3.31	yiaH	-7.62	yjbM	4.77	ymgC	-2.68	yqeH	3.01		
yhbN	-2.97	yiaM	2.16	yjbO	-10.8	ymgD	-4.51	yqeK	2.57		
yhbP	-2.47	yiaO	2.51	yjbQ	-2.38	ymgE	7.25	yqfB	-2.7		
yhbQ	-3.1	yibG	4.17	yjcB	-4.59	ymgG	-8.65	yqgC	-2.13		
yhbS	-2.25	yibK	-2.19	yjcD	-3.17	ymiA	-2.38	yqgE	-4.1		
yhbT	-2.12	yibN	-2.21	yjcE	-3.55	ymjA	-4.83	yqgF	-4.03		
yhbU	-4.43	yibT	2.45	yjcF	4.31	ymjC	-2.25	yqhA	-3.06		
yhbV	-3.66	yicC	-2.41	yjcO	-3.75	ynbC	-2.02	yqiB	-3.06		
yhbW	-3.08	yicE	-2.7	yjdI	3.16	yncA	-3.41	yqiC	-3.94		
yhbY	-8.63	yicH	-2.44	yjdJ	3.34	yncN	-2.54	yqjA	-3.26		
yhcB	-2.42	yicM	-2.19	yjdK	4.98	yneG	-2.85	yqjB	-2.79		
yhcC	-6.66	yicN	-4.43	yjdL	-4.57	yneH	-3.88	yqjI	-2.01		
yhcH	2.23	yicS	-3.21	yjdO	2.67	yneI	-2.42	yraL	-3.14		
yhcM	-2.55	yidC	-16.42	yjdP	-12.3	yneJ	-3.43	yraM	-3.53		
yhcN	-3.93	yidD	-24.52	yjeE	-3.59	ynfA	-2.77	yraP	-5.38		
yhcO	17.25	yidF	-9.73	yjeF	-2.56	ynfB	-3.16	yraQ	-9.83		
yhdA	-4.81	yidH	-2.34	yjeI	-2.25	ynfD	-5.7	yraR	-2.16		
yhdH	-3.3	yidI	-5.87	yjeM	-2.12	ynfE	-3.72	yrbA	-4.46		
yhdL	-3.79	yidQ	-5.68	yjeO	-2.99	ynfK	-3.63	yrbB	-8.53		
yhdT	-5.17	yidR	-5.02	yjeS	-2.04	ynfL	-2.23	yrbC	-6.85		
yhdU	-2.28	yidX	-3.12	yjeT	-3.31	yniC	-3.82	yrbD	-3.63		
yhdW	-2.01	yieI	-2.11	yjfC	-2.91	yniD	-2.79	yrbE	-6.5		
yheS	-2.34	yifK	-3.63	yjfL	-2.6	ynjE	-3.85	yrbF	-6.83		
yheT	-2.41	yifL	-2.15	yjfM	-2.12	ynjF	-2.65	yrbG	-4.43		
yhfA	-2.13	yigG	2.17	yjfY	2.65	yoaA	-2.53	yrbK	-2.29		
yhfK	-2.58	yigI	-4.43	yjfZ	-2.52	yoaB	-2.81	yrfF	-11.05		
yhfY	-2.4	yigP	-3.99	yjgF	-2.85	yoaD	-2.64	yrfG	3.62		
yhfZ	-2.08	yigZ	-2.23	yjgJ	-2.05	yoaE	-6.06	yrhB	-2.05		
yhgE	2.25	yihA	-2.36	yjgM	-2.06	yoaF	-4.25	ysaA	-2.91		
yhgN	-3.35	yihE	-4.86	yjgN	3	yoaH	-4.31	ysaB	-21.55		

List of differentially expressed genes in the 50 □g/mL samples.

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
aaeA	2.45	ansA	3.42	asnC	2.02	caiA	2.11	clpP	2.11	cysC	5.16
aaeX	2.62	ansP	2.61	asnS	3.27	caiB	2.99	cls	2.21	cysD	4.36
aas	2.45	apaG	2.78	aspA	2.35	caiF	4.07	coaA	4.43	cysE	2.59
aat	3.21	apaH	2.15	aspC	5.01	can	2.55	coaD	2.08	cysG	3.27
abgT	2.24	aphA	3.82	aspS	2.49	carA	9.1	coaE	3.29	cysH	5.72
accA	2.29	appA	4.23	asr	9.75	carB	7.82	cobB	3.23	cysJ	5.65
accB	2.12	appB	6.79	astA	2.06	cbl	3.34	cobC_2	2.48	cysK	4.73
accD	2.54	appC	6.59	astD	2.15	cbpA	3.7	copA	5.95	cysM	3.51
aceA	2.97	apt	2.21	atoA	2.2	cbpM	2.43	corC	3.27	cysN	4.47
aceB	4.03	araH	2.08	atoC	2.16	cbrC	3.28	cpdA	2.21	cysP	3.17
aceE	3.42	araJ	2.43	atoE	2.72	ccmA	2.26	cpdB	2.56	cysS	2.63
aceF	3.57	arcB	2.38	atpA	3.38	ccmB	2.4	crcA_1	2.41	cysU	2.86
aceK	2.62	argA	3.39	atpB	2.71	ccmC	3.73	crcA_2	3.4	cysW	2.74
ackA	3.72	argB	3.02	atpC	3.37	ccmD	5.72	crcB_1	3.36	dacA_1	3.33
acnA	2.72	argC	3.36	atpD	3.15	ccmE	3.49	crcB_2	2.53	dacA_2	3.52
acnB	2.67	argD	5.15	atpE	2.74	ccmF	3.64	crl	2.43	dadA	3.03
acpH	2.27	argE	3.55	atpF	3.43	ccmG	2.09	crp	3.49	dadX	2.38
acpP	3.13	argG	3.4	atpG	3.46	ccmH	2.99	crr	3.1	dam	2.57
acpT	2.3	argH	2.66	atpH	3.39	cdaR	2.01	csgA	2.87	damX	2.46
acrA	2.09	argI	3.6	atpI	2.38	cedA	3.02	csgB	2.98	dapA	2.06
acrB	2.15	argO	2.47	avtA	2.15	chbB	2.25	csgC	2.28	dapB	4.47
acrR	2.11	argP	3.02	azoR	2.49	chbF	2.8	cspA	2.18	dapD	4.4
acs	2.54	argR	2.48	baeR	2.77	chbG	2.1	cspC	6.33	dapE	2.28
adhE	5.73	argT	2.38	barA	2.61	chbR	3.55	cspD	2.7	dapF	2.48
adhP	2.1	arnA	2.2	basR	3	cheB	2.34	cspE_1	4.76	dcd	2.42
adiC	9.5	arnB	9.28	basS	2.97	cheW	3.1	cspE_2	5.15	dcm	2.16
adk	3.03	arnC	8.05	bcp	4.91	cheY	3.83	cspH	-3.15	dcp	3.56
agaA	2.46	aroA	4.91	betT	2.06	cheZ	2.15	csrA	2.43	dcuA	2.87
agaB	2.3	aroB	2.42	bfd	6.8	cho	2.05	cueO	5.22	dcuB	2.74
agaI	2.18	aroC	2.91	bfr	8.19	chpA	3.21	cueR	2.08	dcuC_1	2.51
agp	3.42	aroD	4.69	bglA	3.44	chpR	4.89	cusF_1	-3.75	dcuC_2	3.08
ahpC_1	5.11	aroE	2.29	bglG	6.82	cirA	6.76	cusR_1	2.39	dcyD	2.63
ahpC_2	5.35	aroF	6.05	bglH	2.06	citA_1	2.01	cusR_2	2.27	ddlA	2.66
ahpF_1	3.48	aroG	4.88	bglJ	2.27	citB_1	2.98	cusS_1	2.17	def	2.9
ahpF_2	3.88	aroH	9.75	bioA	3.67	citB_2	3.12	cutA	5.4	degQ	2.21
aidB	2.42	aroK	3.2	bioB	10.12	citC_1	2.58	cvpA	2.33	degS	2.04
ais	9.31	aroL	5.02	bioC	4.37	citC_2	2.26	cvrA	2.35	deoD	2.3
alaS	2.27	aroM	2.57	bioD	6.68	citD_1	3.41	cyaY	2.96	deoR	2.16
aldB	2.53	aroP	8.48	bioF	7.77	citD_2	3.58	cybB	2.33	dfp	2.38
alkA	2.28	arsB	3.52	bipA	2.12	citE_1	3.34	cycA	2.67	dgkA	2.88
allC	2.36	arsC	5.05	birA	2.45	citE_2	3.02	cydA	2.03	dgoR	2.74
allD	3.52	arsR	3.54	bisC	2.79	citF_1	4.09	cydC	3.24	dgsA	2.34
allR	3.1	artI	5.48	bolA	3.21	citF_2	3.94	cydD	2.23	dgt	2.81
alsK	2.45	artJ	8.48	borD_1	3.42	citG_1	3.86	cynS	3.21	dhaK	2.64
amiC	2.17	artM	3.19	borD_2	3.29	citG_2	3.56	cynX	3.17	dhaL	3
amn	4.18	artP	4.22	btuB	3.18	citT_1	4.84	cyoA	2.08	dhaM	2.93
ampD	2.33	artQ	3.42	btuD	2.02	citT_2	4.68	cyoB	2.3	dinB	2.17
ampE	2.91	ascG	2.39	btuE	2.68	citX_1	3.11	cyoC	2.56	dinD	2.41
ampG	2.1	asd	4.48	btuF	2.49	citX_2	3.5	cyoD	2.31	dinI	4.23
ampH	2.82	asmA	2.36	btuR	2.4	cld	2.26	cyoE	3.22	dinJ	2.06
amtB	2.36	asnA	4.63	cadB	2.67	clpA	2.15	cysA	4	dipZ	3.46
amyA	2.96	asnB	3.98	cadC	2.95	clpB	3	cysB	4.08	djlA	2.17

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
djlB_1	2.51	entS_2	14.44	fiu	2.1	frdC	2.42	glmU	2.66	gshA	2.43
djlC_2	2.02	envC	2.18	fklB	2.76	frdD	2.04	glnA	7.81	gshB	2.02
dksA	2.01	envY_1	2.87	fkpA	2.65	fre	2.06	glnB	4.19	gsiA	2.72
dld	5.78	eptA	3.13	fldA	3.3	frmA	2.03	glnD	2.17	gsiB	2.82
dmsA	2.7	eptB	2.16	flgA	6.32	frr	3.03	glnE	3.02	gsiC	2.32
dmsB	3.03	essD_2	2.21	flgB	13.03	frsA	2.04	glnG	2.91	gsk	2.13
dmsC	2.49	eutE	2.03	flgC	8.19	frwB	2.76	glnH	9.47	gspG	2.37
dmsD	2.54	eutP	4.59	flgD	9.3	frwC	2.27	glnK	4.15	gspK	2.06
dnaB	2.14	evgA	4.56	flgE	9.16	fsaA	2.44	glnL	4.35	gspL	2.25
dnaC	2.17	exbB	6.08	flgF	7.62	fsaB	2.04	glnP	5.36	gspM	2.34
dnaJ	2.41	exbD	5.12	flgG	6.41	fsr	3.07	glnQ	4.54	gspO	2.81
dnaK	3.25	exoX	2.49	flgH	6.86	ftn	4.42	glnS	2.55	gss	3.3
dnaQ	2.58	fabA	2.74	flgI	4.33	ftsB	2.11	gloA	3.68	gst	2.08
dnaX	2.44	fabB	3.33	flgJ	2.78	ftsW	2.15	gloB	3	guaA	2.98
dps	3.6	fabG	2.19	flgK	3.16	fucR	3.19	glpA	-2.33	guaB	2.38
dsbA	2.77	fabI	2.85	flgL	3.36	fucU	2.28	glpE	2.32	guaC	2.75
dsbG_1	4.34	fabR	2.05	flgN	3.01	fumA	2.97	glpQ	2.14	guaD	2.22
dsbG_2	2.68	fabZ	2.37	flhA	2.63	fumB	2.85	gltA	4.64	gyrA	2.67
dsdC	2.19	fadB	2.15	flhD	5.51	fur	2.73	gltB	6.69	hcaB	2.07
dsdX	2.48	fadL	2.68	fliA	3.84	fxsA	3.66	gltD	7.04	hcaT	2.12
dsrB	2.41	fadR	2.1	fliD	2.75	gabP	2.29	gltI_1	6.34	hcr	2.09
dtd	2.14	fbaA	4.72	fliE	4.12	gabT	4.49	gltI_2	5.32	hdeA	28.09
dusC	2.01	fbaB	5.77	fliF	5.94	gadA	17.49	gltJ_1	2.52	hdeB	29.08
dut	2.14	fdhD	2.24	fliG	3.91	gadB	25.6	gltJ_2	3.17	hdeD	14.86
eaeH	2.28	feaB	2.71	fliH	4.81	gadC	11.11	gltK_1	2.44	hdhA	2.47
eamA	2.25	fecA	4.74	fliI	4.42	gadE	23.04	gltK_2	3.25	hemA	2.34
ebgR	2.41	fecB	2.56	fliJ	4.43	gadW	5.69	gltL_1	4.76	hemB	2.1
ecnB	3	fecC	2.28	fliK	3.73	gadX	5.82	gltL_2	3.94	hemC	2.25
eda	2.53	fecI	7.84	fliL	5.85	galF	3.65	gltX	2.84	hemD	2.35
efeO	4.18	fecR	4.06	fliM	5.87	galR	2.21	glxK	2.18	hemE	2.54
efp	2.42	fepA_1	9.79	fliN	7.77	galU	2	glyA	4.57	hemH	2.65
elaA	3.67	fepA_2	11.29	fliO	2.84	gapA	5.35	glyS	2.6	hemL	2.89
elaB	4.2	fepB_1	5.23	fliP	2.86	garK	2.13	gmhB	3.71	hemN	2.34
elaD	2.73	fepB_2	6.7	fliQ	2.49	garR	2.1	gmk	2.11	hemY	2.07
elbA	3.63	fepC_1	5.36	fliR	2.39	gatC	2.15	gmr	2.52	hflD	2.26
elbB	2.61	fepC_2	3.99	fliS	3.53	gcvA	2.91	gnd	4.24	hflK	2.04
emrK	5.52	fepD_1	6.73	fliT	3.17	gcvH	6.88	gnsA	2.92	hflX	2.43
emrY	3.58	fepD_2	7.96	fliY	4.88	gcvP	5.28	gntR	3.02	hfq	3.2
emtA	2.45	fepG_1	3.86	fliZ	5.21	gcvR	5.59	gntY	2.73	hinT	2.46
eno	5.01	fepG_2	3.01	fmt	2.18	gdhA	7.32	gor	2.44	hipA	2.38
entA_1	2.47	fes_1	7.04	fnr	3.45	gfcA	2.55	gph	2.8	hipB	2.25
entB_1	7.09	fes_2	12.27	focA	3.74	ggt	2.1	gpmA	5.67	hisA	3.99
entB_2	5.63	ffh	2.04	folA	2.01	glcC	2.08	gpsA	2.42	hisB	3.72
entC_1	15.22	fhlA	2.22	folD	4.2	glcG	2.87	gpt	2.4	hisC	3.12
entC_2	14.94	fhuA	4.02	folE	4.42	gldA	3.2	greA	2.51	hisD	2.82
entD_1	3.59	fhuB	2.5	folK	2.58	glgA	2.85	groL	3.42	hisF	3.91
entD_2	2.6	fhuC	2.41	folM	2.04	glgB	2.7	groS	3.44	hisG	2.85
entE_1	8.86	fhuD	2.53	folP	2.42	glgC	2.88	grpE	3.29	hisH	3.68
entE_2	8.49	fhuE	2.36	folX	3.28	glgP	2.88	grxA	6.66	hisI	3.35
entF_1	2.78	fhuF	6.81	fpr	2.59	glgS	3.51	grxB	4.06	hisJ	4.57
entF_2	2.16	fieF	2.23	frc	2.79	glk	2.1	grxC	2.32	hisM	5.17
entS_1	13.82	fimZ	4.38	frdA	2.3	glmM	2.16	grxD	2.69	hisP	5.43

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
hisQ	4.11	ilvN	2.81	livF	4.06	map	2.7	modF	2.22	napH	4.47
hisS	2.22	imp	2.62	livG	3.84	marR	2.53	moeA	2.48	narG	4.85
hlyE	2.01	infA	3.35	livH	2.1	matA	4.59	moeB	2.11	narH	3.23
hmp	10.02	insA-3	2.51	livJ	2.79	matB	4.22	mog	2.04	narI	2.38
hns	2.57	insB-4	2.01	livK	2.92	mazG	3.08	motA	2.36	narJ	3.56
hokC	-2.58	insE-2	2.69	livM	3.18	mdaB	2.21	mpl	2.15	narK	2.79
holA_2	2.03	insG	2.02	lldR	2.7	mdh	3.05	mppA	2.61	narL	2.31
hpf	2.74	insI-3	3.79	lnt	2.09	mdoD	2.75	mqo	2.37	narP	2.25
hpt	2.46	insJ	3	lolB	2.3	mdoG	2.32	mqsR	5.47	narV	2.15
hscC_2	2.29	intB	2.11	lolC	2.62	mdtH	3.11	mraY	2.54	ndh	5.65
hslO	2.25	intD	2.56	lolD	2.95	mdtL	2.03	mrdB_2	2.17	ndk	2.07
hslR	2.46	iraP	3.87	lolE	2.73	menB	2.68	mrp	2.56	nemA	3.19
hslU	3.3	iscA	2.66	lon	2.6	menC	2.36	mscL	4.48	nfnB_1	2.59
hslV	3.28	iscR	2.67	lpcA	2.76	menF	2.43	mscS	2.75	nfnB_2	2.64
htpG	2.93	iscS	2.5	lpd	2.91	mepA	2.39	msrA	3.03	nfo	2.77
htpX	2.1	iscU	2.93	lpp	2.98	metA	10.5	msyB	2.44	nfsA	2.35
htrE	2.43	ispB	2.57	lpxA	2.11	metB	7.54	mtlA	3.59	nhaA	2.31
hupA	3.35	ispF	2.02	lpxH	3.63	metC	5.97	mtlD	2.75	nhaB	2.39
hupB	2.7	ispG	2.53	lpxM	2.1	metE	6.89	mtr	6.15	nhaR	2.09
hyaA	4.96	ispU	2.17	lpxP	2.06	metF	8.54	mug	2.57	nhoA	3.38
hyaB	4.21	ivbL	3.08	lrp	3.19	metG	3.5	murB	3.1	nikA	2.93
hyaC	3.01	katE	2.43	lsrC	2.11	metH	4.28	murC	2.12	nikB	4.11
hyaD	3.86	katG	3.55	lsrF	2.48	metI	4.27	murD	2.89	nikC	4.88
hyaE	2.06	kbl	2.12	lsrG	2.59	metJ	3.15	murE	3.41	nikD	4.69
hyaF	2.26	kch_1	3.55	lsrR	2.96	metK	4.84	murF	2.96	nikE	3.23
hybC	2.18	kdgR	2.87	ltaE	2.71	metL	5.32	murI	2.21	nikR	3.33
hybD	2.08	kdgT	2.05	luxS	4.54	metN	5.07	mutM	2.35	nirB	4.24
hycB	2.1	kdsA	2.97	lysA	3.56	metQ	6.99	mutS	2.12	nirC	6.63
hycC	2.59	kdsB	2.44	lysC	6.34	metR	5.5	mutT	2.26	nirD	4.54
hycD	3.65	kdtA	2.18	lysP	3.23	mfd	3.49	mviM	2.58	nlpA	4.68
hycE	4.56	kefB	2.11	lysS	2.66	mglB	2.92	nac	2.7	nlpC	2.31
hycF	4.6	kefC	2.18	lysU	3.09	mgsA	2.64	nadA	4.02	nlpI	2.16
hycG	5.7	kefF	2.42	maeA	3.5	mhpA	2.33	nadB	3.02	nmpC_1	3.51
hycH	4.59	kgtP	2.46	mak	2.3	mhpR	2.54	nadC	3.17	norR	2.18
hycI	4.08	ksgA	2.42	malE	43.8	miaA	2.45	nadD_1	3.46	npr	2.72
hydN	2.71	lacA	2.77	malF	6.67	minC	2.06	nadD_2	3.5	nrdA	2.09
iaaA	3.16	lacI	2.08	malG	3.74	minD	2.9	nadE	4.97	nrdB	2.25
ibpA	2.9	lacY	2.08	malI	2.37	minE	3.2	nadR	2.23	nrdE	2.04
ibpB	3.49	lamB	23.63	malK	22.39	mipA	2.27	nagA	2.94	nrdH	4.23
icd	3.95	ldcA	2.1	malM	4.69	mlrA	2.24	nagB	2.96	nrdI	2.89
iclR	3.64	ldhA	2.15	malP	6.99	mltB	2.83	nagC	3.73	nrdR	2.55
idnD	2.59	ldrA	2.17	malQ	2.13	mltC	2.45	nagD	2.69	nrfA	2.67
idnR	2.54	ldrB	-2	malS	5.2	mngR	3.3	nagK	2.46	nrfC	2.57
idnT	2.04	leuS_1	2.33	malT	2.9	mnmA	2.3	nagZ	2.47	nrfD	2.65
ihfA	2.22	leuS_2	2.33	malX	2.71	moaA	3.07	nanE	3.46	nrfF	2.04
ihfB	2.05	lexA	3.4	malY	3.09	moaB	3.48	nanR	3.02	nsrR	2
ileS	2.01	lgt	2.59	malZ	2.93	moaC	5.69	napA	7.94	nudB	2.73
ilvA	3.72	ligB	2.12	manA	2.39	moaD	2.51	napB	6.91	nudC	2.64
ilvB	2.47	lipA_1	2.81	manX	3.16	moaE	4.16	napC	3.06	nudE	2.31
ilvC	3.17	lipA_2	2.73	manY	3.44	mobA	2.67	napD	3.26	nudF	3.05
ilvE	3.93	lipB_1	3.34	manZ	3.67	mobB	2.19	napF	2.94	nudG	2.2
ilvM	3.05	lipB_2	2.91	maoC	2.19	modE	2.27	napG	5.41	nudH	3.05

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
nudJ	2.6	pepD	4.32	pphA	2.24	putA	2.88	rffM	2.96	rpsU	2.7
nuoA	2.77	pepE	2.66	ppiA	2.18	putP	2.22	rffT	2.25	rraB	4.24
nuoB	3.18	pepN	3.27	ppiB	6.91	puuA	2.5	rhaR	2.28	rrmA	2.14
nuoC	3.87	pepP	2.94	ppk	4.3	puuC	2.06	rhaS	2.34	rsd	3.59
nuoE	4.39	pepQ	3.31	pptA	2.93	puuP	2.29	rhlB	2.44	rsgA	2.1
nuoF	4.32	pepT	2.62	ppx	3.92	puuR	2.04	rho	2.58	rsmB	2.36
nuoG	4.77	pfkB	3.86	pqiB	2	pykA	3.38	rhtB	2.94	rsmC	2.08
nuoH	4.44	pflA	3.96	pqqL	2.01	pykF	3.61	rhtC	3.25	rspB	2.31
nuoI	4.66	pflB	5.07	prc	2.32	pyrB	9	ribA	2.26	rssA	2.36
nuoJ	4.78	pflC	2.39	prfA	2.3	pyrC	5.47	ribC	3.31	rstA	4.53
nuoK	3.75	pfs	2.93	prfB	2.51	pyrD	4.04	ribE	2.66	rstB	4.42
nuoL	4.04	pgaB	2.13	priA	2.03	pyrE	3.68	ribF	2.05	rtn	3.4
nuoM	4.87	pgaC	2.42	prkB	2.03	pyrG	2.39	rihA_1	2.76	rumA	2.58
nuoN	4.27	pgaD	2.83	prlC	3.14	pyrH	2.49	rihA_2	3.4	rutG	2.44
nupC	2.89	pgi	5.02	prmB	3.07	pyrI	5.09	riml	2.37	rutR	2.99
nusB	2.15	pgk	3	proA	2.13	pyrL	2.63	rimJ	3.41	ruvB	2.14
ompA	2.93	pgl	2.92	proB	2.11	qor	3.03	rimL	2.85	ruvC	2.01
ompC	2.8	pgm	2.23	proC	2.73	qseB	2.53	rlpA_1	2.87	rzoR	-2.62
ompF	9.84	pgpA	2.08	proQ	2.33	queC	3.48	rlpA_2	2.89	rzpD_1	3.73
ompT_1	3.34	pgpB	2.07	proS	2.55	queF	2.31	rlpB_1	2.21	rzpD_2	2.32
ompT_2	3.61	pgsA	2.97	prsA	3	radA	2.03	rluC	2.12	rzpR	3.43
ompX	2.46	pheA	2.75	psiF	2.1	rarD	2.22	rluD	2.21	sapA	2.76
oppA	5.87	pheS	3.36	pspA	2.18	rbsA	2.57	rluE	2.34	sapB	3.75
oppB	4.68	pheT	2.71	pspB	2.09	rbsB	4.25	rluF	3.08	sapC	2.34
oppC	4.3	phnH	2.93	pspC	2.22	rbsC	3.33	rmf	3.96	sapD	2.55
oppD	4.27	phnJ	2.13	pspD	2.43	rbsD	3.64	rna_1	2.43	sbcB	2.55
oppF	4.77	phoH	2.73	pspE	2.19	rbsK	2.93	rna_2	2.56	sbcC	2.86
orn	2.99	phoP	3	pspF	2.28	rcsA	2.67	rnb	3.27	sbcD	2.67
osmY	3.23	phoQ	2.29	pssA	2.61	rcsD	2.03	rnc	2.61	sbmC	2.88
otsA	2.32	phr	2.33	pstA	2.02	rcsF	3.55	rnd	2.35	sbp	2.7
otsB	2.23	pioO	2.52	pstS	2.17	rdgC	3.3	rne	3.09	sdaA	2.23
oxc	2.25	plsC	2.42	pta	4.31	recN	2.07	rng	2.21	sdhA	2.12
oxyR	2.18	pmbA	3.38	pth	3.3	recO	2.05	rnhA	2.87	sdhB	2.15
paaX	2.16	pmrD	2.97	ptsA	2.11	recR	2.62	rnk_2	2.73	sdiA	3.3
paaY	2.88	pncA	3.56	ptsG	2.41	relA	2.28	rnt	2.16	secA	2.39
pal	2.66	pncB	2.52	ptsH	2.96	rfaB	3.51	rpe	2.41	secB	3.1
panB	2.97	pntA	2.83	ptsI	2.72	rfaC	3.8	rpiA	3.21	secM	2.36
panC	3.62	pntB	2.24	ptsN	2.48	rfaD	3.78	rpiB	3.49	selB	2.95
panD	3.94	pnuC	2.87	ptsP	2.73	rfaE	2.65	rpiR	2.93	seqA	2.86
parC	2.29	polA	3.47	purA	2.1	rfaF	2.83	rplI	2.55	serA	7.24
parE	2.18	potB	2.22	purB	5.6	rfaG	2.75	rplT	2.58	serB	2.39
pbpG	3.88	potC	2.51	purC	4.52	rfaI	3.05	rplU	2.02	serC	5.98
pck	2.26	potD	3.48	purD	3.78	rfaJ	4.22	rpmA	2.09	serS	2.72
pcm	2.05	potF	3.34	purE	4.77	rfaP	2.42	rpmE	2.48	setC	2.96
pdhR	2.06	potG	2.22	purF	4.7	rfaQ	3.1	rpoB	2.67	sfmC	3.1
pdxA	2.2	potH	2.63	purH	4.68	rfaY	4.03	rpoC	2.68	sfmD	2.24
pdxB	2.39	potI	2.02	purK	4.04	rfaZ	3.86	rpoN	2.2	sfsA	2.01
pdxH	2.09	poxB	3.48	purL	5.17	rfe	2.17	rpoZ	2.62	sfsB	2.41
pdxJ	2.33	ppa	3.96	purM	3.5	rffD	2.1	rpsA	2.11	sgbH	3.13
pdxK	3.06	ppc	6.53	purN	2.74	rffE	2.09	rpsB	2.08	shiA	5.87
pepA	2.02	ppdB	2.41	purT	2.98	rffG	2.56	rpsG	2.12	skp	2.25
pepB	2.23	ppdC	2.37	purU	3.48	rffH	2.66	rpsT	2.4	slp	9.52

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
slyA	2.59	tas	2.28	trpB	53.43	wzb	2.06	ybaA	2.15	ybgE	2.13
slyB	3.17	tatA	2.61	trpC	45.61	wzc	2.31	ybaB	2.33	ybgF	2.68
slyD	3.13	tatB	2.24	trpD	60.78	wzxC	2.92	ybaJ	2.29	ybgH	2.69
smf	2.1	tatC	2.12	trpE	46.74	wzyE	2.27	ybaK	2.46	ybgI	2.35
smg	2.44	tatE_1	2.52	trpR	2.63	wzzE	2.05	ybaM	2.5	ybgJ	2.11
smpB	3.05	tatE_2	3.06	trpS	3.14	xapR	2.17	ybaN	2.79	ybgK	2.17
sodA	5.47	tauA	3.25	trxA	3.22	xdhC	2.24	ybaP	3.22	ybgO	2.02
sodB	3.13	tauB	2.43	trxB	2.77	xerC	2.58	ybaQ	3.34	ybgP	2.93
sohA	2.32	tauC	2.46	trxC	3.1	xseB	2.08	ybaS	2.62	ybgS	2.46
sohB	3.26	tdcC	2.4	tsf	2.2	xthA	2.33	ybbC	3.45	ybhA	2.27
solA	4.48	tdcE	2.51	tsx	2.03	xylA	2.11	ybbL	3.18	ybhB	3.68
soxR	3.17	tdcG	2.14	ttcA	2.05	xylE	2.22	ybbM	3.62	ybhC	2.87
soxS	2.86	tdh	2.41	ttk	2.26	yaaA	2.8	ybbN	3.16	ybhI	2.11
speA	2.55	tdk	3.29	tufA	2.32	yaaY	2.61	ybbO	2.23	ybhK	2.1
speB	2.05	tehA	5.11	tus	2.34	yacC	2	ybbW	3.14	ybhL	2.57
speC	4.62	tehB	4.29	tyrA	7	yacF	2.12	ybcJ	2.29	ybhM	2.76
speD	3.17	tesA	2.09	tyrB	6.5	yacG	2.79	ybcQ	2.43	ybhN	2.49
speE	2.45	tesB	2.05	tyrR	2.24	yacH	2.25	ybcS_1	4.82	ybhP	6.45
speG	2.45	thiC	24.67	tyrS	2.06	yadC	3.3	ybcS_2	3.01	ybhT	2.89
spoT	2.36	thiD	4.07	ubiB	2.06	yadG	2.29	ybdB_1	3.29	ybiA	3.09
spoU	2.09	thiE	28.59	ubiC	2.05	yadK	2.38	ybdD_1	4.83	ybiB	2.22
sppA	2.97	thiF	28.87	ubiE	2.56	yadL	2.41	ybdD_2	2.22	ybiC	3.31
spr	4.81	thiG	30.48	ubiG	3.27	yadR	3.17	ybdF_2	2.04	ybiI	3.01
spy	3	thiH	24.88	ubiH	2.29	yaeB	2.58	ybdH_1	5.04	ybiJ	2.99
sra	6.28	thiK	2.12	ubiX	2.62	yaeH	3.79	ybdH_2	4.89	ybiM	2.4
srlD	2.13	thiL	2.08	udk	3.5	yaeJ	2.19	ybdJ_1	2.2	ybiU	2.99
srlE	3.83	thiM	4.19	ugd	2.51	yaeP	2.21	ybdJ_2	3.34	ybiV	2.3
ssb	2.43	thiS	32.41	ulaR	2.56	yaeQ	2.34	ybdL_1	5.42	ybjD	2
sscR	2.49	thrA	2.93	umuD	3.31	yafC	2.5	ybdL_2	5.93	ybjI	2.39
sseA	2.08	thrB	2.62	ung	2.33	yafJ	2.31	ybdM_1	2.81	ybjJ	2.11
sseB	2.09	thrC	3.33	upp	3.19	yafK	3.24	ybdM_2	4.23	ybjM	2.62
sstT	4.01	thrS	2.67	uraA	3.12	yafM	2.36	ybdO_2	2.67	ybjN	2.41
ssuA	4.07	thyA	3.39	ushA	2.96	yafN	2.39	ybdR_1	2.54	ybjT	2.46
ssuB	2.76	tiaE	2.29	uspA	2.41	yafO	2.21	ybdZ_1	2.24	ybjX	3.26
sthA	2.47	tig	2.31	uspB	2.62	yafP	2.23	ybeA_2	2.29	ycaC	5.92
stpA	2.91	tktA	3.86	uspE	2.55	yafS	2.78	ybeB_1	2.23	ycaD	2.15
sucA	4.07	tktB	2.12	uvrC	2.61	yafT	2.07	ybeB_2	2.62	ycaK	2.02
sucB	3.6	tldD	2.55	uvrY	3.67	yafZ	-2.1	ybeD_1	3.08	ycaL	2.13
sucC	4.16	tolA	2.09	uxaC	2.57	yagR	2.18	ybeD_2	3.06	ycaN	2.12
sucD	4.39	tolC	3.96	valS	2.1	yagT	2.8	ybeL_1	2.67	ycaO	2.62
sufA	5.08	tonB	4.36	viaA	2.46	yagU	3.22	ybeL_2	2.66	ycaP	2.57
sufB	2.68	topA	2.58	visC	2.09	yahB	2.01	ybeQ_1	3.49	ycaR	2.91
sufD	2.13	torC	2.22	vsr	2.02	yahD	7.35	ybeQ_2	2.41	ycbG	2.52
sufI	2.42	torY	2.03	wcaC	2.12	yaiA	4.27	ybeR_2	2.46	ycbK	2.38
suhB	2.44	torZ	2.28	wcaD	4.26	yaiE	3.47	ybeY	3.34	ycbL	2.3
sulA	2.37	tpiA	4.69	wcaE	2.59	yaiI	2.73	ybeZ	3.81	ycbQ	2.03
syd	2.34	tpx	4.53	wcaF	2.86	yaiL	2.97	ybfA	2.47	ycbU	2.13
talA	4.77	treA	2.36	wcaI	2.25	yajC	3	ybfD	2.15	ycbV	2.53
talB	3.81	treF	2.38	wcaK	2.09	yajD	3.6	ybfF	2.72	ycbW	2.46
tam	2.88	trkD	2.65	wcaL	2.33	yajG	3.27	ybfM	3.02	ycbZ	2.03
tap	3.09	trmI	2.29	wrbA	7.66	yajL	2.16	ybgA	3.73	yccA	2.57
tar	2.95	trpA	69.11	wza	2.13	yajQ	2.5	ybgC	2.58	yccE	2.14

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
yccF	2.34	ycjQ	2.53	ydhW	3.45	yecC	2.66	yeiN	2.28	yfhL	4.2
yccJ	7.22	ycjR	2.06	ydhX	4.02	yecE	2.1	yeiP	3.08	yfhM	2.02
yccK	2.25	ycjW	2.66	ydhY	3.03	yecF	2.61	yeiR	2.77	yfiA	2.05
yccU	3.22	ycjX	3.11	ydhZ	2.1	yecH	2.89	yeiT	2.62	yfiD	3
yccX	3.54	ycjZ	2.08	ydiA	3.92	yecJ	3.49	yeiU	3.74	yfiF	2.24
ycdT	2.4	ydaM	2.26	ydiB	5.42	yecM	2.57	yejA	2	yfiQ	2.37
ycdU	3.02	ydbC	3.8	ydiE	3.99	yecN	2.61	yejH	2.69	yfjB	2.92
ycdW	2.58	ydbD	2.89	ydiH	4.13	yecO	2.39	yejK	2.02	yfjD	2.38
ycdX	2.91	ydbH	2.37	ydiI	4.02	yecP	2.16	yejL	2.39	yfjF	2.6
ycdY	3.01	ydbJ	2.56	ydiJ	6.45	yecR	3.56	yejM	2.16	yfjG	2.29
yceA	2.4	ydbK	2.53	ydiN	2.59	yecS	3.2	yfaD	2.38	yfjX	2.73
yceF	2.42	ydbL	2.51	ydiO	2.76	yedA	2.83	yfaO	6.09	ygaD	2.27
yceH	2.62	ydcC	4.3	ydiP	3.74	yedD	3.09	yfaW	2.43	ygaF	2.48
yceI	3.6	ydcD	2.29	ydiR	2.1	yedE	3.38	yfaY	3.57	ygaP	3.46
yceJ	2.12	ydcH	4.82	ydiU	2.09	yedF	3.47	yfaZ	2.13	ygaT	3.7
yceQ	2.41	ydcK	3.44	ydiV	2.01	yedK	2.06	yfbJ	2.76	ygaU	2.36
ycfD	2.1	ydcL	3	ydiY	2.41	yedP	2.42	yfbO	2.43	ygaV	4.78
ycfH	2.05	ydcQ	2.9	ydiZ	4.58	yedV	2.67	yfbP	2.61	ygaW	6.23
ycfL	2.47	ydcR	2.53	ydjA	2.72	yedW	2.79	yfbT	3.52	ygbA	8.16
ycfM	2.55	ydcX	3.69	ydjF	3.34	yedX	3.01	yfbU	3.68	ygbE	4.36
ycfQ	2.1	ydcY	2.75	ydjG	2.42	yedZ	2.81	yfbV	2.16	ygbI	2.01
ycfR	4.38	ydcZ	2.97	ydjH	3.54	yeeA	2.1	yfbW	2.05	ygcB	2.62
ycfS	2.25	yddA	3.45	ydjN	2.66	yeeD	4	yfcA	2.21	ygcE	2.2
ycfT	2.85	yddE	2.3	ydjO	3.4	yeeE	3.07	yfcD	2.24	ygcF	3.24
ycgB	3.93	yddG	3.82	ydjX	4.53	yeeF	2.36	yfcE	2.29	ygcL	2.17
ycgE	2.49	yddV	4.33	ydjY	6.49	yeeI	3.2	yfcF	3.34	ygcO	-2.51
ycgF	2.2	ydeE	2.81	ydjZ	4.89	yeeJ	2.23	yfcI	2.05	ygcP	2.85
ycgJ	3.06	ydeH	2.02	yeaD	5.32	yeeN	7.2	yfcO	2.26	ygcQ	3.17
ycgK	2.14	ydeI	2.28	yeaE	2.39	yeeX	5.04	yfcV	2.3	ygcU	2.42
ycgL	3.39	ydeJ	2.07	yeaG	4.73	yeeZ	2.33	yfdF	3.62	ygdD	2.1
ycgM	2.83	ydeM	3.27	yeaH	3.74	yegD	2.67	yfdO	2.69	ygdE	2.15
ycgN	2.94	ydeN	2.23	yeaK	2.87	yegE	2.32	yfdP	7.92	ygdG	2.02
ycgX	2.52	ydeO	3.77	yeaL	2.29	yegH	2.08	yfdQ	4.53	ygdH	3.25
ycgY	2.43	ydeP	2.4	yeaN	2.67	yegK	2.07	yfdT	3.58	ygdL	2.16
ychF	2.88	ydeQ	2.87	yeaO	3.33	yegP	5.91	yfdV	2.73	ygdQ	2.24
ychJ	4.01	ydeR	2.32	yeaP	2.39	yegR	2.4	yfdY	2.56	ygdR	2.41
ychN	2.89	ydfG	4.95	yeaQ	6.26	yegT	2.3	yfdZ	2.75	ygeA	3.7
yciA	2.47	ydfH	2.46	yeaS	3.15	yegU	2.13	yfeA	2.24	ygfB	3.25
yciE	2.51	ydgA	3.36	yeaT	3.2	yegW	2.13	yfeC	2.29	ygfI	2.07
yciF	3.37	ydgD	2.3	yeaZ	2.91	yehD	2.93	yfeD	3.03	ygfU	2.16
yciH	2.39	ydgH	3.91	yebC	2.42	yehE	3.25	yfeX	2.06	ygfY	2.07
yciI	4.3	ydgI	2.11	yebE	2.37	yehK	2.68	yffB	2.54	ygfZ	2.64
yciK	2.5	ydgJ	3.72	yebF	2.55	yehS	2.39	yffH	2.51	yggD	2.22
yciO	2.42	ydhF	2.68	yebG	3.42	yehT	3.17	yfgA	2.63	yggJ	2.11
yciQ	2.02	ydhJ	2.1	yebK	2.24	yehU	2.12	yfgB	2.09	yggL	2.97
yciT	3	ydhM	4.39	yebO	3.9	yehZ	2.19	yfgD	2.48	yggN	2.02
yciU	2.12	ydhO	2.46	yebQ	2.23	yeiA	3.31	yfgG	4.3	yggP	2.24
yciW	4.85	ydhQ	2.49	yebR	4.09	yeiB	2.5	yfgH	2.98	yggS	2.19
yciX	3.15	ydhR	4.44	yebU	2.2	yeiE	2.28	yfgM	2.09	yggT	2.08
ycjF	3.61	ydhS	2.16	yebV	3.71	yeiG	3.15	yfhA	2.34	yggU	2.19
ycjG	2.03	ydhU	2.75	yebY	2.18	yeiI	2.08	yfhJ	2.25	yggW	2.53
ycjM	2.24	ydhV	4.82	yecA	2.3	yeiM	2.38	yfhK	2.14	yggX	3.2

ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold
yghA	2.04	yhhA	2.23	yifL	3.8	yjeT	2.16	ynbD	2.53	yphF	2.44
yghB	2.64	yhhI	2.15	yigB	4.22	yjfP	2.53	ynbE	5.16	yphH	2.27
yghO	2.06	yhhW	3.08	yigF	2.45	yjfZ	2.59	yncA	4.36	yqaA	2.18
yghQ	2.77	yhhX	6.91	yigL	2.63	yjgA	2.25	yncB	2.13	yqaB	2.27
yghS	2.53	yhhY	4.15	yigZ	2.01	yjgB	2.03	yncC	2.46	yqcE	2.03
yghU	3.64	yhiD	19.57	yihA	2.64	yjgF	2.68	yncE	6.5	yqeF	3.08
ygiF	2.91	yhiF	6.29	yihD	4.1	yjgH	8.53	yncG	5.35	yqeI	11.39
ygiH	2.24	yhiM	6.94	yihE	2.11	yjgI	14.85	yneE	3.73	yqeJ	4.65
ygiN	3.33	yhiQ	2.73	yihF	2.75	yjgJ	5.44	yneH	2.38	yqfA	2.45
ygiP	2.41	yhjA	2.3	yihG	2.43	yjgK	5.2	yneI	4.07	yqfB	2.6
ygiQ	2.09	yhjC	2.3	yihI	2.85	yjgM	2.03	yneL	8.14	yqgB	3.09
ygiS	2.7	yhjE	3.24	yihL	2.88	yjgR	2.22	ynfA	2.59	yqgC	2.65
ygiT	4.19	yhjG	2.38	yihM	2.88	yjhA	2.02	ynfB	2.68	yqgD	2.34
ygiW	2.67	yhjH	2.01	yihO	2.46	yjhT	2.75	ynfC	3.15	yqgE	2.73
ygiZ	-3.07	yhjX	21.96	yihS	2.58	yjhV	3.02	ynfE	2.15	yqgF	2.06
ygjG	3.76	yhjY	2.06	yihV	2.28	yjjA	2.9	ynfF	4.16	yqhA	2.17
ygjI	2.36	yiaA	3.78	yihW	2.18	yjjG	2.36	ynfG	3.07	yqiA	2.04
ygjJ	2.57	yiaB	4.69	yiiE	2	yjjK	2.78	ynfH	2.18	yqiB	2.41
ygjM	2.22	yiaC	3.52	yiiF	3.55	yjjP	2.46	ynfO	3.74	yqiC	2.42
ygjP	2.89	yiaF	2.69	yiiG	2.18	yjjQ	2.83	yniA	2.14	yqiG	2.18
ygjR	3.39	yiaG	8.45	yiiM	2.1	yjjU	3.05	yniB	2.7	yqiH	2.25
ygjV	2.19	yiaH	2.08	yiiQ	3.64	yjjV	2.22	yniC	3.48	yqiJ	2.01
yhaH	3.62	yiaJ	2.1	yiiR	2.72	yjjW	2.07	yniD	2.6	yqjD	2.2
yhaI	2.6	yiaT	2.08	yiiS	2.99	yjjY	2.13	ynjA	4.56	yqjF	2.14
yhaJ	2.44	yiaU	2.91	yiiT	3.7	yjtD	2.07	ynjB	4.54	yqjG	2.42
yhaK	2.12	yibD	2.38	yiiU	2.16	ykgA	3.39	ynjC	2.71	yqjH	5.38
yhaL	2.2	yibF	3.98	yijO	2.49	ykgB	4.89	ynjD	2.25	yraH	3.48
yhaM	2.07	yibJ	3.23	yijP	3.17	ykgC	8.13	ynjE	5.39	yraI	2.55
yhaV	2.53	yibL	2.54	yjaG	2.53	ykgD	2.5	ynjI	2.38	yraL	2.76
yhbJ	2.51	yibN	2.88	yjbA	2.07	ykgG	3.27	yoaA	2.14	yraM	2.62
yhbX	2.76	yibQ	2.08	yjbB	2.32	ykgH	7.17	yoaB	2.83	yraN	3.48
yhbY	2.28	yicC	2.79	yjbD	5.09	ykgI	3.98	yoaC	2.69	yrbA	2.41
yhcB	3.47	yicE	2.17	yjbE	5.62	ykgO	-2.21	yoaF	2.11	yrdA	3.56
yhcF	3.04	yicG	2.19	yjbH	2.25	ylbA	2.85	yoaH	2.35	yrdB	2.79
yhcH	4.44	yicI	2.06	yjbI	3.26	ylbE	2.04	yobA	2.27	yrdC	2.17
yhcM	3.67	yicJ	2.75	yjbJ	2.71	yliE	3.44	yobD	3.32	yrdD	2.13
yhcO	2.99	yicL	3.47	yjbM	3.78	yliF	2.09	yobF	5.85	ysgA	3.77
yhdH	2.71	yicN	2.04	yjbO	2.39	yliG	2.15	yobG	3.09	ytfA	4.91
yhdJ	2.37	yicO	2.02	yjbQ	3.08	yliJ	2.03	yobH	3.05	ytfB	2.69
yhdW	2.54	yicR	2.07	yjbR	2.53	yliL	3.3	yoeB	2.23	ytfE	9.7
yhdZ	2.31	yidA	3.7	yjcD	2.48	ymdA	3.69	yohC	3.97	ytfF	2.01
yheN	2.4	yidB	2.31	yjcH	-2.15	ymdB	2.42	yohF	2.03	ytfG	3.93
yheO	2.69	yidK	2.39	yjcO	2.49	ymdF	4.31	yohH	2.37	ytfH	4.26
yheV	3.4	yidL	4.48	yjdA	2.49	ymgA	4.28	yohM	2.11	ytfP	2.82
yhfA	3.67	yidZ	2.22	yjdC	3.22	ymgB	6.11	yohO	3.42	ytjA	2.99
yhfL	5.94	yieG	2.6	yjdF	2.26	ymgE	5.57	yojI	3.69	ytjB	2.8
yhfT	2.24	yieH	3.43	yjdK	3.06	ymgF	3.59	ypdA	2.37	yzfA	4.3
yhfW	2.03	yieI	2.5	yjdO	2.06	ymgJ	2.71	ypdB	3.05	zapA	2.48
yhfX	3.24	yieK	2.01	yjeH	2.45	ymiA	3.07	ypdH	-4.25	zitB	2.63
yhfY	3.55	yieP	2.22	yjeI	3.21	ymjA	3.17	ypeA	2.25	znuA	2.39
yhfZ	2.12	yifE	3.3	yjeJ	2.02	ynaI	2.56	ypfN	2.57	zur	3.42
yhgF	2.14	yifK	2.37	yjeM	2.27	ynaJ	2.8	yphA	3.94	zwf	2.9

List of differentially expressed genes in the 75 □g/mL samples.

Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID	Fold	ID
aceB	2.06	cysN	2.46	fucP	-2.11	iscU	2.02	paaI	-2.01	trpC	14.06
adhE	2.61	cysP	2.64	fumB	2.14	ivbL	2.09	pepD	2.02	trpD	19.45
agaB	-2.99	cysU	2.24	gatA	5.74	kdpA	-2.18	pflB	2.92	trpE	22.29
agp	2.17	cysW	2.01	gatB	-3.33	kgtP	2.2	pgi	2.72	trxC	2.1
ahpC_1	2.13	dapB	2.77	gcvH	3.23	lamB	22.7	phoH	2.51	tyrA	4.18
ahpC_2	2.19	dapD	2.77	gcvP	2.6	luxS	2.74	ppc	4.74	tyrB	3.62
ais	3.58	dcuB	2.11	gcvR	2.64	lysC	5.69	pta	2.12	uspF	-2.22
amn	2.34	ddpX	-2.12	gdhA	4.11	malE	24.09	purB	2.19	wcaF	2.58
aqpZ	-2.44	dld	2.41	glnA	3.11	malF	5.71	purL	2.32	yadN	2.13
argB	2.02	emrY	2.12	glnH	3.16	malG	4.34	putA	2.49	yafO	2.24
argD	2.28	eno	2.3	glnK	2.28	malK	24.28	puuA	2.18	yafZ	-2.66
argE	2.2	entC_1	2.48	glnL	2.11	malM	4.26	puuB	2.15	yahD	2.43
arnB	4.4	entD_1	2.83	gloA	2.72	malP	6.13	puuC	2.06	yaiS	-3.13
arnC	2.03	etp	2.05	gltA	3.38	malS	4.36	puuP	2.74	ybbC	2.43
aroA	2.69	fadB	2.19	gltB	4.61	malZ	2.31	pyrB	2.5	ybcV_2	=
aroF	4.65	fepA_1	2.08	gltD	4.43	matA	2.21	pyrC	2.5	ybdL_1	2.12
aroG	2.65	fepA_2	2.18	gltI_1	3.26	metA	3.6	pyrI	2.33	ybdL_2	2.47
aroH	3.63	fepB_1	2.04	gltI_2	3.57	metB	2.35	racC	=	ybdZ_2	2.07
aroL	2.36	fimZ	2.07	gltJ_1	2	metC	3.56	rbsB	2.29	ybfQ	-2.6
aroP	3.99	flgA	3.19	gltK_2	2.13	metE	2.36	rbsD	2.2	ybiC	2.02
arsB	2.36	flgB	8.63	gltL_1	2.63	metH	2.02	rnpA	-2.28	ybiV	2.03
arsR	3.58	flgC	8.47	gltL_2	2.86	metI	2.01	rpmD	-2.06	ycaK	2.38
artI	2.47	flgD	7.1	gpmA	2.2	metQ	3.16	rsd	2.63	ycfR	3.71
artJ	3.2	flgE	8.86	grxA	2.68	metR	2.39	rutE	-2.09	ycgF	2.05
asd	2.78	flgF	6.24	grxB	2.17	modF	2.29	rzoR	7.27	ycgL	2
asnA	2.62	flgG	5.65	gsiA	2.2	mqsR	3.39	sdiA	2.73	yciF	3
asnB	2.68	flgH	4.77	gspC	2.05	msrA	2.18	serA	4.02	yciW	3.17
aspC	2.2	flgI	4.27	hcaC	2.02	mtr	2.75	serC	3.31	ycjM	3.06
bcp	2.62	flgJ	3.19	hdeD	2.01	nadE	2.76	shiA	2.44	ycjN	3.22
bfr	2.93	flhA	2.04	hisJ	2.3	nikB	2.07	speC	2.1	ycjO	2.91
bglG	-2.53	flhB	2.07	hisM	2.63	nikD	2.24	sstT	2.32	ycjP	3
bioB	2.1	flhD	2.48	hisP	3.02	nikE	2.02	ssuB	2.02	ycjQ	2.82
bioF	2.07	fliA	2.57	hisQ	2.14	nlpA	3.05	sucA	2.11	ycjR	2.54
carA	3.07	fliE	3.87	hmp	3.9	nohB_1	2.48	sucB	2.05	ycjS	3.04
carB	3.32	fliF	4.18	hokE_2	-2.55	nuoF	2.02	sucC	2.22	ycjT	2.66
chpR	2.08	fliG	2.55	hycE	2	nuoG	2.16	sucD	2.29	ycjW	2.1
citG_1	2.12	fliH	3.09	hycG	2.5	nuoI	2.13	tauA	3.03	ydaT	-2.01
coaA	2.03	fliI	2.96	hycI	2.01	nuoK	2.05	tauB	2.45	ydbL	3
copA	3.67	fliJ	4.48	hypC	-2.18	nuoL	2.01	tdcA	-3.12	yddV	3
cspC	2.61	fliL	3.21	iaaA	2.7	nuoM	2.24	thiC	4.13	ydfG	2.16
cspH	2.27	fliM	4.64	icd	2.25	nuoN	2.07	thiE	4.29	ydfR	3.23
cueO	3.09	fliN	5.33	idnD	2.21	ompF	5.47	thiF	4.93	ydfZ	-2.24
cusF_1	-2.76	fliO	2.71	ilvC	2.98	ompL	3.12	thiG	5.32	ydgJ	2.03
cynS	2.3	fliS	3.16	insE-3	8.98	ompT_1	2.44	thiH	5.06	ydhM	2.41
cysA	2.66	fliT	2.05	insE-4	3.34	ompT_2	2.53	thiS	3.97	ydhR	2.14
cysB	2.02	fliY	3.21	insF-1	4.79	oppA	2.81	thrB	2.19	ydhV	3.92
cysC	2.65	fliZ	2.88	insF-2	4.18	oppB	2.63	thrC	2.6	ydhW	2.29
cysD	2.52	flxA	=	insF-3	4.41	oppC	2.52	torY	2.05	ydhX	2.24
cysH	2.99	focA	2.13	insJ	4.02	oppD	2.6	torZ	2.15	ydhY	2.27
cysJ	3.02	folE	2.27	insN-1	2.23	oppF	2.73	tpx	2.25	ydhZ	2.09
cysK	2.99	fruK	-2.31	iraP	2.59	osmB	-2.06	trpA	12.46	ydiI	3.09
cysM	2.09	ftn	3.26	iscR	2.06	paaG	-2.22	trpB	13.16	ydiJ	3.81

Fold	ID	Fold	ID
ydiZ	3.11	ykiB	3.84
ydjO	2.01	ymdA	3.93
ydjY	3.24	ymgF	-3.31
ydjZ	2.21	ymiA	2.8
yeaD	2.2	yncA	2.36
yeaG	2.69	ynfF	2.72
yeaQ	2.83	ynfG	2.46
yebR	2.2	ynjE	2.52
yedX	2.09	yoaC	2.13
yeeD	2.02	yoaG	2.76
yeeX	2.02	yobF	2.82
yehC	-2.65	yohM	2.23
yehD	2.1	ygeJ	-4.5
yehE	3.79	ygeK	3.15
yeiL	2.79	ygqD	-2.17
yfbO	2.32	ygjH	2.66
yfbU	2.1	ygsA	2.15
yfcZ	-2.15	ytfE	5.21
yfdP	2.14	ytfH	2.28
yfdQ	2.09	zntA	-2.22
yfjX	-2.74		
ygbA	2.54		
ygdH	2.09		
ygeF	-3.91		
ygeI	-11.76		
ygiL	2.03		
ygiS	2.18		
ygiT	2.97		
yhcA	2.16		
yhfY	2.59		
yhhX	2.91		
yhhY	2.05		
yhiS	-4.71		
yhjX	-3.36		
yiaG	2.94		
yiaW	-2.12		
yibF	2.1		
yidD	-2.33		
yidG	2.38		
yihR	2.47		
yjbD	3.28		
yjbE	4.23		
yjcF	-2.14		
yjfL	2.14		
yjgH	4.88		
yjgI	7.9		
yjgJ	3.66		
yjgK	2.65		
ykgB	2.02		
ykgC	4.02		
ykgD	2.16		
ykgO	14.83		