Excessive folate synthesis in *Escherichia coli* and its influence on *Caenorhabditis elegans* ageing

Bhupinder K. Virk

A thesis submitted to Durham University in accordance with the requirements for the degree of Doctor of Philosophy

September 2013

School of Biological and Biomedical Sciences

Durham University

ABSTRACT

A previous discovery showed that a spontaneous mutant in the *aroD* gene of the RNAi bacteria HT115(DE3), caused a significant increase in *C. elegans* longevity. In this thesis, I aimed to confirm this finding and attempt to determine whether chemical inhibition of folate synthesis in other *E. coli* strains would also increase *C. elegans* lifespan, and uncover the mechanism via which inhibited folate synthesis increases animal lifespan.

In order to confirm that the *aroD* mutant *E. coli* increase lifespan I investigated effects of using different media types. Data show that the *aroD* mutant *E. coli* shows variable effects on *C. elegans* lifespan with different media types due to the varying folate content of media used. I found that the *aroD* mutant *E. coli* effect on lifespan was maximal using a high purity agar with peptone. I then attempted to mimic the effect on other *E. coli* bacterial strains, such as OP50 commonly used as the *E. coli* food source for *C. elegans*, by using sulfamethoxazole as a chemical intervention to inhibit folate synthesis. Sulfamethoxazole increased *C. elegans* lifespan in a dose dependent manner. Interestingly, even though SMX is used as an antibiotic, the highest concentration used did not appear to decrease growth of the *E. coli* lawn substantially. Liquid chromatography coupled to mass spectrometry was used to measure and confirm the folate levels in both *E. coli* and *C. elegans*.

In order to uncover the specific mechanism by which inhibition of folate synthesis increases *C. elegans* lifespan, I needed to distinguish whether folate status of *E. coli* itself, folate status of *C. elegans*, or an interaction of folate status in both organisms was responsible for the increase in lifespan. The drug methotrexate (MTX) and the *C. elegans gcp-2.1* mutant were used to investigate the effect of inhibiting the worm

folate cycle, and impeding animal folate uptake. Results suggest that animal folate status does not play a role in the extended longevity. SMX and kanamycin have similar but not additive effects on lifespan, suggesting a common mechanism for both drugs. This is surprising as kanamycin prevents bacterial proliferation, whilst SMX does not. Bacterial accumulation assays suggested that bacterial accumulation may be a marker of ageing not a cause in this system.

Together the data presented here show that it may be possible to use chemical interventions to treat the mammalian gut flora, inhibiting excessive microbial folate synthesis and potentially slow ageing, without disrupting microbial ecology.

This one's for you Dad

ACKNOWLEDGEMENTS

I would like to thank my supervisor David Weinkove, for giving me this opportunity and for all the guidance and support over the past 4 years; I know it hasn't been easy.

I would not have been able to complete this PhD without the support of my fellow Worm lab members, who have provided useful discussion and feedback, and who have seen me through the best and worst science days. My particular gratitude goes to those of you who have contributed to this study: Adelaide Raimundo, Nikolin Oberleitner, Goncalo Correia, Inna Feyst, Marta Ciplinski.

I would like to thank Jackie Mosely, David Dixon, Ian Cummins and Mark Skipsey for their help and guidance with regard to Mass Spectrometry.

Last but not least I would like to thank my family for their support and belief in me whilst I pursue this project.

DECLARATION

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

……

Bhupinder Virk

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMPK, AMP-activated protein kinase

CFU, colony forming units

CHES, N-Cyclohexyl-2-aminoethanesulfonic acid

CoA, coenzyme A

CR, caloric restriction

DHF, dihydrofolate

DHFR, dihydrofolate reductase

DHPS, dihydropteroate synthase

DMG, dimethylglycine

DR, dietary restriction

dTMP, Thymidine monophosphate

dUMP, deoxyuridine monophosphate

ESI TOF, electrospray ionisation time-of-flight

FEB, folate extraction buffer

Glu_x, poly glutamate with x representing the number of glutamate moieties

HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HPLC, high pressure liquid chromatography

HPLC-MS, high pressure liquid chromatography coupled to mass spectrometry

IIS, insulin/IGF-1 signaling pathway

LC-MS, liquid chromatography coupled to mass spectrometry

MS, methionine synthase

MS-MS, tandem mass spectrometry

MTHF, methylenetetrahydrofolate reductase

MTX, methotrexate

NGM, nematode growth media

OD_{600}, optical density at absorbance 600nm

P, phosphate

PABA, para-aminobenzoic acid

PAB-glu, para-aminobenzoyl-glutamate

PCR, polymerase chain reaction

PEP, phosphoenolpyruvate

PHB, para-hydroxybenzoic acid

PI3K, phosphoinositide-3-OH kinase

Pte, Pteroic acid

Q-TOF, quadrupole time-of-flight

ROS, reactive oxygen species

SAH, s-adenosylhomocysteine

SAM, s-adenosylmethionine

SFG, sulfaguanidine

SHK, shikimate

SHMT, serine hydroxymethyltransferase

SMX, sulfamethoxazole

SOD, superoxide dismutase

THF, tetrahydrofolate.

TOR, Target of Rapamycin

TS, Thymidine synthase

UMFA, unmodified folic acid.

5m-THFGlu_5, 5-methyl-tetrahydrofolate with 5 glutamates

CHAPTER 1

INTRODUCTION

1.1 Overview

Ageing is something that we have all felt the effects of, either in ourselves or in loved ones. Researchers in the field of ageing have suggested many different theories and ideas about why we age, and the direct causes of ageing. Implications have been made about the importance of diet and the role of gut microbes in many chronic diseases and ageing. Gut microbes provide animals with essential nutrients and vitamins that animals themselves cannot synthesis, such as folates.

The well characterised model organism *Caenorhabditis elegans* has long been used for the study of ageing, due to its short lifespan and ease of maintenance. In the laboratory, *C. elegans* is maintained on lawns of *Escherichia coli*. Together these two model organisms provide a system in which interactions between host and microbe, and also the effect of diet on health can be more closely studied.

1.2 Why do we age?

Many people do not consider ageing as a disease, but rather something inevitable that happens to all of us after a certain period of time. As people grow older, they notice changes in the way in which their bodies work, a decrease in fertility, muscles weaken, and bones become more prone to fracture. Along with these changes the risks of getting certain diseases such as cancer and diabetes increases with age, as does mortality (Kirkwood & Austad 2000). It is not just humans that age, almost all organisms and biological systems show deleterious effects of ageing or senescence, be

it tissue degradation or loss of normal functionality or efficiency. However, there are some exceptions, species that do not appear to show signs of increased mortality or a reduction in fertility with increasing age, such as *Hydra*, suggesting that ageing is not a trait that effects all organisms (Finch 1994; Kirkwood & Austad 2000). Not all species age gradually either, the Pacific salmon ages all at once after their one chance of reproduction in life has passed, this is thought to be driven by sex hormones (Kirkwood 2008).

1.3 Evolutionary theories of ageing

If some organisms do not show any apparent signs of ageing, this implies that ageing is not a universally programmed trait (Kirkwood & Austad 2000; Kirkwood 2005). With such negative effects on individuals, the question arises as to why ageing would evolve at all. The field of ageing research is vast, and much work has gone into trying to determine why and how ageing has evolved. Understanding why ageing has evolved, provides us with an insight to the nature of the genes that are involved in the ageing process, and how they can shape the relationship between reproduction and survival in life history (Kirkwood 2008). It has been suggested that the diversity of life history patterns observed in nature, is an effect of natural selection not being strong enough to control intrinsic deterioration of the body (Kirkwood 2005; Kirkwood 2008).

In the literature there are three main theories regarding the evolution of ageing. The mutation accumulation theory first suggested by Medawar, suggests that the selective force on individuals progressively decreases with age. Therefore in a wild population, by an age when survival levels have become extremely low, the selective pressures on

the few surviving individuals has also become very weak. The selective force at this late stage is too weak to act against any germ-line mutations that have deleterious effects later in life. This may then act to delay the onset of such deleterious alleles to ages where they would have very little selective pressure to purge them. High levels of extrinsic mortality would suggest early reproduction to be beneficial, and after reproduction there would be little or no selective pressure on deleterious effects later in life. (Medawar 1952; Kirkwood & Austad 2000; Kirkwood 2005; Kirkwood & Melov 2011).

Williams then further developed Medawar's theory and suggested that pleiotropic genes that had positive effects early in life would be selected for even if they exhibited deleterious effects later in life, as selective pressure is theoretically stronger earlier in life (Williams 2001; Kirkwood & Austad 2000; Kirkwood 2005). As fitness can be assessed by magnitude of the effect and by the probability of surviving to be affected, then a small benefit earlier in life may be selected for in spite of a late acting significantly negative effect. This theory is known as the antagonistic pleiotropy theory (Kirkwood & Austad 2000).

Although the mutation accumulation and antagonistic pleiotropy theories have been well established in the literature with regards to evolutionary theories of ageing, there is little positive evidence, and much evidence to the contrary with regards to the feasibility of mutation accumulation (Kirkwood & Austad 2000; Kirkwood 2008). A *Drosophila* study by Service *et al* suggested that mutation accumulation may act alongside trade-offs (Service et al. 1988).

Other studies on *Drosophila* appeared to provide evidence and support for mutation accumulation (Hughes & Charlesworth 1994; Charlesworth & Hughes 1996), however further research has raised doubts (Promislow & Tatar 1996; Shaw et al. 1999). With respect to the antagonistic pleiotropy theory there have been relatively few confirmed individual genes that have been shown to have the type of antagonistic pleiotropy originally proposed by Williams (Leroi et al. 2005; Kirkwood & Melov 2011).

The third theory for the evolution of ageing is the disposable soma theory, which looks at the optimal allocation of metabolic resources, mainly energy, between reproduction and somatic maintenance for maximum Darwinian fitness (Kirkwood 1977; Kirkwood & Holliday 1979; Kirkwood & Austad 2000; Kirkwood 2005). The theory suggests that in the wild, somatic maintenance is required to keep the individual in appropriate physiological condition as long as the individual has a good chance of surviving unfavourable conditions. An example of this can be found in wild mice. Approximately 90% of wild mice die in the first year; therefore investment of energy into mechanisms for survival beyond this age will only benefit 10% of the population. Almost all of the processes needed for somatic maintenance, including DNA repair, require a high amount of energy. The main cause of death in mice is the cold in winter; mice are unable to maintain thermogenesis showing that resources are limited. The disposable soma theory suggests that the mouse will benefit by allocating any spare resources into thermogenesis, rather than repair or reproduction. If this were to happen, then the lack of somatic repair would lead to an accumulation in damage leading to ageing (Kirkwood & Austad 2000; Kirkwood 2005). The disposable soma theory focuses on the evolution of optimal levels of cell maintenance, and makes specific predictions about the biology of ageing. One prediction is that ageing results

from lifelong accumulation of unrepaired molecular and cellular damage through evolved limitations in repair functions and somatic maintenance. Other predictions are that longevity is controlled in principle by genes that regulate levels of somatic maintenance and repair functions, and that immortality of the germ line may require higher levels of maintenance and repair in germ cells as compared with somatic cells. Furthermore disposable soma predicts that the mechanisms behind cellular and molecular ageing are strongly influenced by chance. Also that the allocation of resources to repair and maintenance is determined by evolutionary optimization and the allocation strategy may need to be adaptive to respond to individual variations of circumstance during the organism's life cycle. These predictions help to bring a mechanistic specificity to the evolutionary theory of ageing (Kirkwood 2008).

1.4 Mechanistic theories of ageing

Evolutionary theories of ageing do not necessarily help us to understand the direct causes or mechanisms behind the ageing process. There are many changes in an individual that occur during the ageing process, reminding us how complex ageing is. This complexity has resulted in a plethora of theories that suggest specific cellular and molecular causes of ageing. There are too many theories of ageing to discuss in detail, a review in 1990 reported over 300 theories of ageing (Medvedev 1990). Here I will discuss a few of the more popular and key theories of ageing.

1.4.1 Cellular senescence/Telomere loss theory

In 1965 Hayflick found that almost all human cells have a limited number of cell doublings that they are capable of in culture (Hayflick 1965). This means there is a characteristic number of divisions before a cell enters a terminally arrested state

(Weinert & Timiras 2003; Kirkwood 2005; Campisi & Fagagna 2007). This led to the theory that this intrinsic limit also limits organism lifespan. This restriction on the number of divisions a cell can make is thought to be due to loss of telomeres during replication events (Harley 1991; Harley & Vaziri 1992; Levy et al. 1992; Olovnikov 1996; Kirkwood 2005; Weinert & Timiras 2003). It has been suggested that immortal cell lines like germ line cells or stem cells express telomerase which acts to extend telomeres (Cech 2004). It is thought that senescent cells may create a hazardous environment and therefore may have an effect on neighbouring cells (Krtolica & Campisi 2002). A possible cause for this effect on neighbouring cells may be due to the fact that senescent cells have been shown to have higher expression of pro-inflammatory cytokines (Weinert & Timiras 2003). Whilst the relevance of the hayflick limit to *in vivo* cellular senescence has been questioned (Rubin 2002), studies on baboons have shown that potentially more than 15% of skin cells in ageing animals are senescent (Herbig et al. 2006). This effect was not observed in muscle, indicating that perhaps the level to which senescent cells contribute to ageing differs by tissue and organism (Jeyapalan & Ferreira 2007).

1.4.2 Oxidative stress theory

Oxidative stress has long been thought to be involved in determining the rate of ageing (Harman, 1956). The theory suggests that ageing is caused by exposure to reactive oxygen species (ROS), which then leads to the oxidative damage in the cell, which accumulates with age. This links ageing to mitochondrial metabolism, since superoxide (O_2^-) production by the electron transport chain is the greatest source of ROS in cells (Brand, 2000). Studies show that there are increased levels of oxidant-damaged DNA and proteins in older cells (Harman 1992; Harman 1993; Beckman &

Ames 1998; Weinert & Timiras 2003). Whilst there is convincing evidence showing that oxidative damage accumulates in ageing cells, it has not been shown that the oxidative damage is a direct cause of ageing. The oxidative stress theory seems to be consistent with the underlying idea that animals with fast metabolism live shorter than those with a slower one (the so-called "rate of living" theory). Selection of different metabolic rates in different organisms leads to different rates of O_2^- production and therefore, different rates of ageing (Gems, 2009; Sohal and Weindruch, 1996). However, this long-held view of a direct relationship between metabolic rate and ROS production is now seen as naïve, given the extent to which they are uncoupled (Brand, 2000).

The effects of the superoxide dismutase (SOD) mimetics EUK-8 and EUK-134, which catalyze the disposal of O_2^- on *C. elegans* lifespan, have been investigated (Gems and Doonan, 2009). It was found that 2mM paraquat shortens lifespan and generates O_2^- and that certain concentrations of EUK-8 can rescue this shortened lifespan. These same concentrations of EUK-8 however, are not capable of extending lifespan in the absence of paraquat. This strongly suggests that O_2^- levels do not limit worm lifespan in usual conditions (Keaney et al., 2004). Treatment with higher doses of SOD mimetics actually shortens *C. elegans* lifespan and EUK-8 has also been shown to cause increases in oxidative damage in *E. coli* (Gems and Doonan, 2009). It is possible that strongly increased SOD activity actually increases overall ROS by increasing levels of H_2O_2 (Buettner et al., 2006).

1.4.3 Gene regulation theory

The gene regulation theory of ageing suggests that senescence is due to a change in gene expression with age. Microarray studies have shown that the expression of many genes change with time (Reinke 2002; Weinert & Timiras 2003). Microarrays allow researchers to create a genetic profile of model organisms, which can then be used as a comparison for interventions that may slow or promote the ageing process. This type of research, at the level of the genome, may lead researchers to key genes in which changes in expression level play a role in the ageing process, thus providing detail about specific mechanisms behind senescence (Weinert & Timiras 2003). Microarray studies have been used to examine gene expression in mice, with results showing very little variation in gene expression in some tissues of ageing mice (Weindruch et al. 2001; Weindruch et al. 2002).

1.4.4 Somatic mutation theory

It has been shown that the rate of DNA mutation in somatic cells is much higher than in gametic cells (Curtis 1963). It has also been suggested that the rate of spontaneous somatic cell DNA mutation increases with age (Kirkwood 2005). These somatic mutations can accumulate with time, and it has been shown that there are larger amounts of DNA damage in older individuals (Vijg 2000). It is yet to be determined however, whether the accumulation of such mutations can cause a large enough effect to cause ageing.

Mice have been cloned for six generations using somatic nuclear transfer, without showing any apparent effects on their rate of ageing (Wakayama et al. 2000). This

study suggests that in mice at least, somatic mutation accumulation is not enough to impact ageing

1.4.5 Theories of systematic ageing

The theories of ageing mentioned above specify a cellular or molecular level cause of ageing. However there are a number of theories of ageing at the individual (organism) level. One example of this in humans is the neuroendocrine theory of ageing. In this theory of ageing, it is hypothesised that changes in neural and endocrine system functions, including communication and responsiveness of body systems to external stimuli and maintenance of optimal functional state can be detrimental. Another theory of ageing at the organism level is the rate of living theory of ageing. This theory is based on the assumption that an individual has a fixed amount of metabolic potential that is available for use throughout life. In this case the faster you use the metabolic resources the faster you senesce, this theory is also known as the live fast, die young theory of ageing (Weinert & Timiras 2003)

1.5 Dietary Restriction

There has been much research providing evidence that it is possible to alter the rate of ageing using specific interventions. One of the most widely known interventions is a type of diet manipulation referred to as dietary restriction (DR). Many studies have shown that restricting diet without malnutrition not only extends lifespan, in some cases a 50% increase has been observed, but it has also been shown to reduce the occurrence of age-dependent diseases in almost all species.

Research shows that there are many methods of inducing DR in *C. elegans* (Lenaerts et al. 2008; Greer & Brunet 2009). It is not known currently, whether the different methods of DR extend lifespan through a single common pathway or whether there are independent mechanisms that are activated, dependent on the method used (Greer & Brunet 2009).

In many organisms, DR appears to increase lifespan in part by down-regulating the nutrient sensor TOR (Target of Rapamycin). Inhibition of TOR elicits autophagy, the recycling of cytoplasmic molecules and organelles (Hansen et al. 2008). A study by Hansen *et al* found that DR and TOR inhibition in *C. elegans* produce an autophagic phenotype. Further, inhibiting genes required for autophagy prevents DR and TOR inhibition from extending lifespan, and despite the rejuvenating effect that autophagy is predicted to have on cells, data show that autophagy is not sufficient to extend lifespan (Hansen et al. 2008).

In *C. elegans* AMP-activated protein kinase (AMPK) is activated by a decrease in energy levels, raising the possibility that AMPK may mediate lifespan extension by DR (Greer et al. 2007). Research has shown that AMPK exerts its effects in part via the FOXO transcription factor DAF-16. FOXO/DAF-16 is required for the positive effects of this DR method on lifespan. Data show that expression of active AMPK in *C. elegans* increases stress resistance and extends longevity in a FOXO/DAF-16 dependent manner (Greer et al. 2007).

DR is often termed caloric restriction (CR) where calorie intake is restricted regardless of the food source (carbohydrate, protein and fat) rather than limiting food as in DR.

This is the case with regards to rodents because daily calorie intake rather than food type has been suggested to be the key determinant of lifespan. This hypothesis resulted from the findings that calorie restriction without reducing protein intake increased lifespan, and also no lifespan extension was observed when rats were fed isocaloric diets with reduced fat or minerals (Mair et al. 2005). However in other experiments, rats fed isocaloric diets with altered nutritional composition showed lifespan extension (Mair et al. 2005). Hence, it seems that reducing the level of ingested calories may not always be critical for life-span extension by DR in rodents.

CR has also been shown to have beneficial effects in non-human primates. A study reported the beneficial effects of a 30% CR in female and male rhesus macaques of a wide age range. Results are similar to that of rodent studies where body weight and fat mass are both reduced, and blood lipids, blood pressure and body temperature are all decreased. These findings could be indicative of a longer lifespan, the study is ongoing (Mattison 2003).

1.6 Diet and ageing

Dietary restriction is one method that has been shown to increase lifespan of animals. There is much literature to provide evidence that diet can affect lifespan, for example the longest living national population are the Japanese, and this is thought to be down largely to their healthy diet of rice, fish and vegetables and lifestyle choices. Controlling ones diet is an option available to many of the world's population.

There is a multibillion dollar market for vitamins and health supplements in the US and increasingly so in the UK with the goal of improving human health. Such

supplements claim to increase both lifespan and health, yet none of them have provided substantial evidence to support the claims that they extend lifespan.

Investigating the relationship between diet and ageing in animals is difficult due to many confounding variables. Factors that affect ageing include environmental factors, complexity of an organism's diet and the heterogeneity of the microbiota present in the gut of animals. All wild individuals have different environments to each other. An example in the human population is the difference between an eastern or western diet. An individual's genetics can also have an effect on the role that diet can play with regards to health. There is evidence to show that Asian immigrants have between a four –fold and six-fold increase in diabetes dependent on country of birth, and a increased susceptibility to cardiovascular type II disease compared to Caucasians (Barnett et al. 2006; Cappuccio 1997; Cappuccio 1998). This increased risk of disease is observed in second generation migrants, suggesting that genetics is involved as these second generation immigrants have been shown to share the same risk factors as their parents and grandparent (Barnett et al. 2006).

Most animals include a wide variety of foods in their diet and therefore the effect of a single dietary component is hard to distinguish from effects that other foods or combinations of foods are causing. Separating beneficial effects of diet on lifespan is therefore difficult due to the varying environment in which all organisms live and their genetic predisposition. Another important factor to consider is that as well as food intake in animals, microbes in the gut of animals can also significantly contribute dietary components and nutrients.

1.7 Gut microbiota and human health

In a human body microbes collectively make up 100 trillion cells, with the number of microbes in the gut lumen outnumbering all of the eukaryotic cells in the human body 10 to 1 (Guarner & Malagelada 2003; Qin et al. 2010). Microbial flora have a symbiotic relationship with animals, in that the microbes assist nutrition by converting ingested food into accessible metabolites and also via the synthesis of essential compounds that animals cannot make themselves such as folic acid and can aid absorption of calcium, magnesium and iron (Miyazawa et al. 1996; Younes & Coudray 2001; Guarner & Malagelada 2003). In humans gut microbes also have important trophic effects on intestinal epithelia and on immune structure and function, and protection of the colonised host against invasion by alien microbes (Guarner & Malagelada 2003). Gut microbiota might also play an essential role in certain pathological disorders, including colon cancer and inflammatory bowel diseases. In spite of this, bacteria also play a part in the promotion of human health. Probiotics and prebiotics are known to have a role in prevention or treatment of some diseases (Qin et al. 2010).

Gut microbes in particular have been shown to influence physiology and nutrition, and have been associated with several chronic diseases such as diabetes (Cani et al. 2007; Wen et al. 2008; Larsen & Vogensen 2010; Musso et al. 2010), cardiovascular disease (Wang et al. 2011) and obesity (Li & Wang 2008; Turnbaugh et al. 2006; Ley et al. 2006) but differentiating between correlation and causality proves to be difficult. One study investigates a gut microbe by product lipopolysaccharide endotoxin. This endotoxin is the only know bacterial product that when subcutaneously infused into mice in a pure form, induces obesity and insulin resistance via an inflammation-

mediated pathway (Fei & Zhao 2013). They isolate an endotoxin-producing bacterium isolated from a morbidly obese human's gut, and place it in germfree mice resulting in induced obesity and insulin resistance in the mice. When the morbidly obese human from which the endotoxin-producing *Enterobacter* was isolated was put on a diet of whole grains, traditional Chinese medicinal foods and prebiotics, the relative abundance of the endotoxin-producing *Enterobacter* decreased. During this time the volunteer lost a significant amount of body weight, and recovered from hyperglycemia and hypertension (Fei & Zhao 2013).

1.7.1 Gut microbial composition

The microbes present in the gut have been shown to be diverse, with 128 different phylotypes being identified in one study (Bik et al. 2006), with many more bacteria yet to be described (Guarner & Malagelada 2003). As well as this diversity, different types of bacteria have been shown to colonise and inhabit different areas of the gut (Figure 1). Colonisation of gut bacteria begins after birth with environmental factors playing an important role, as there are difference in gut composition between infants born in developed countries, and those born in developing countries, as well as differences between infants born in different hospital wards (Guarner & Malagelada 2003). Pioneer bacteria are able to modulate gene expression in host epithelial cells (Hooper et al. 2001), therefore can create a favourable habitat for themselves, and prevent growth of other bacteria that are introduced later. The initial colonisation is thus very relevant to the final composition of the flora in adults (Hooper et al. 2002; Guarner & Malagelada 2003). The genera *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus* are predominant in human beings (Simon & Gorbach 1986), whereas aerobes (facultative anaerobes) such

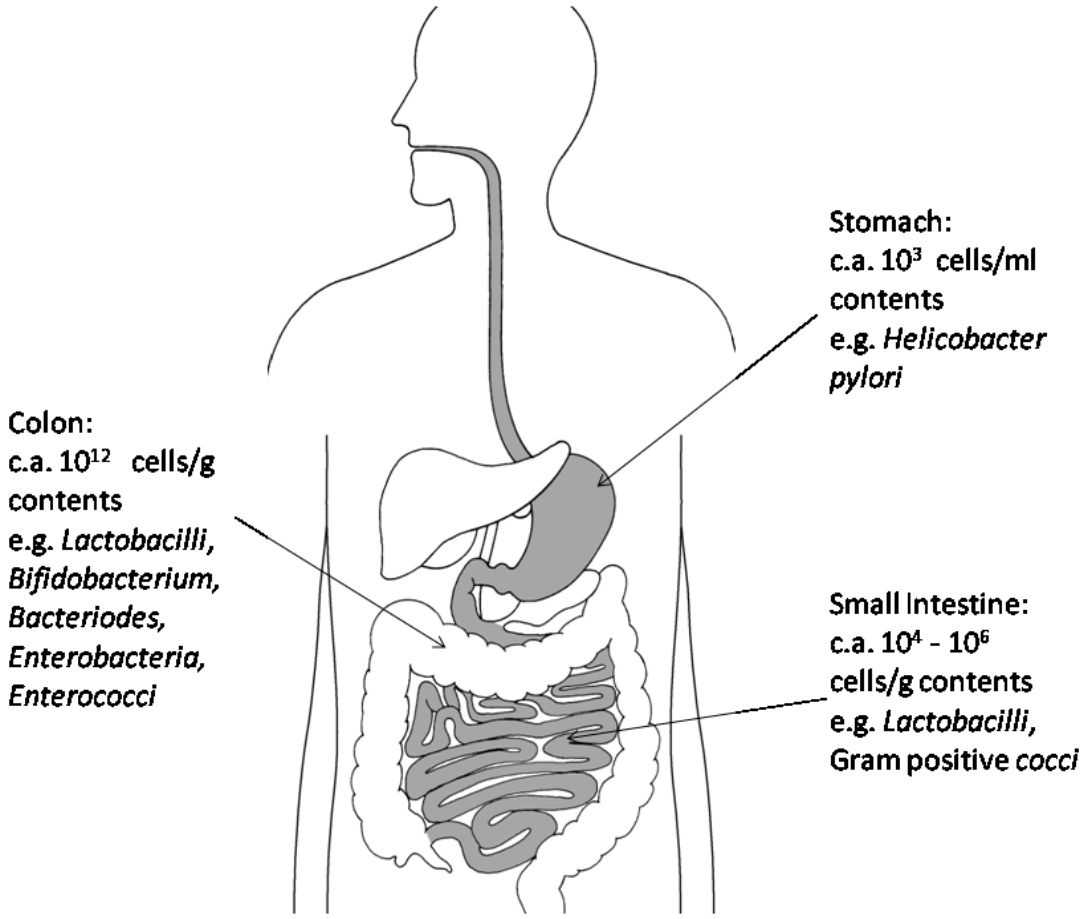


Figure 1: Human intestinal microflora. Image adapted from Dunne 2001. Showing bacterial content and representative species found in the human gut.

as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Proteus*, etc are among the subdominant genera (Simon & Gorbach 1986; Guarner & Malagelada 2003).

It has been suggested that gut microbial composition may be associated with colon cancer. In a human study, the composition of faecal flora of people with differing risks of colon cancer was investigated. High risk of colon cancer was associated with the presence of two particular strains of *Bacteroides*, and low risk appeared to be associated with the presence of two particular strains of *Lactobacillus* and a strain of *Eubacterium* (Moore & Moore 1995). Although the study was not conclusive, it suggests that colonic flora may be a major factor that modulates risk of colon cancer in humans (Moore & Moore 1995; Qin et al. 2010).

1.7.2 Probiotics and prebiotics

Bacteria can be implemented to improve human health. A probiotic is a living microoorganism that when ingested in specific numbers exerts health benefits beyond those of basic nutrition (Guarner & Schaafsma 1998; Gill & Guarner 2004). Prebiotics are non-digestible food ingredients that have beneficial effects on the host by selectively stimulating growth, activity, or both, of just one or a restricted number of bacteria in the colon (Gill & Guarner 2004). Infant formula has been supplemented with probiotics in order to prevent diarrhoeal disease in chronically hospitalised children. *Lactobacillus rhamnosus* strain GG has been used as a prophylaxis of diarrhoea in undernourished children, especially those who have not been breast fed (Guarner & Malagelada 2003).

In yoghurt, bacteria used to start the culture improve digestions of lactose and eliminate symptoms of lactose intolerance in those who do not efficiently absorb lactose (Guarner & Malagelada 2003).

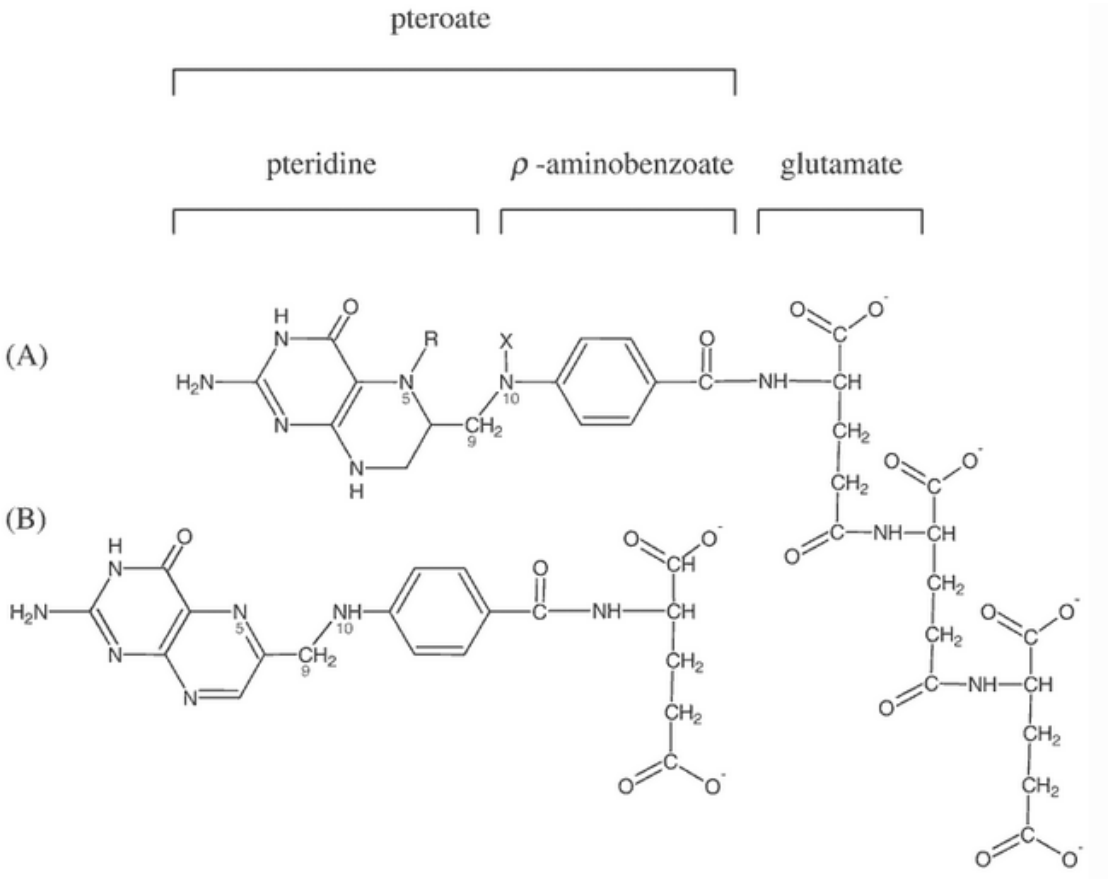
1.8 Folate Synthesis

Folates are B-class water-soluble vitamins, that are required for cell function and development (Bailey & Gregory 1999; Bedhomme et al. 2005). Folate is composed of 'pteroic acid (Pte) covalently bound to a (poly) glutamate chain' (Lu et al, 2007) (Figure 2). The pteroic acid (Pte Glu) can then be modified to synthesize a diverse family of compounds (Figure 2). Folates are involved in the synthesis of many compounds including purines and pyrimidines, methionine and coenzyme A, which are all crucial for one-carbon metabolism (Figure 3) (Reed et al. 2006; Lu et al. 2007; Balamurugan et al. 2007).

De novo, aromatics compounds are synthesised by the shikimic acid pathway. The shikimic acid pathway is responsible for the synthesis of the aromatic amino acids, quinones and para-aminobenzoic acid, a pre cursor of folic acid (Figure 4). However the shikimic pathway is only found in plants and bacteria. Therefore animals rely on their diet and intestinal microbes for aromatic amino acids and folates as they do not possess the required enzymatic activity required for synthesis.

1.9 Folate Metabolism

Folate metabolism is essential in animals, as it is required for the production of purine synthesis, and therefore for DNA and RNA synthesis as well as many other reactions including amino acid metabolism and methylation processes (Shane & Stokstad 1975;



Folate		R	X
Tetrahydrofolic acid (THF)	(H_4PteGlu)	H	H
10-formyl-THF	(10-CHO-H_4PteGlu)	H	CHO
5-formyl-THF	(5-CHO-H_4PteGlu)	CHO	H
5-methyl-THF	(5-CH_3-H_4PteGlu)	CH_3	H
5,10-methenyl-THF	(5,10-CH_2-H_4PteGlu)	=CH-	
5,10-methylene-THF	(5,10-CH^+-H_4PteGlu)	-CH_2-	
7,8-dihydrofolate	(H_2PteGlu)		H

(A) polyglutamyl tetrahydrofolates, (B) Folic acid (PteGlu)

Figure 2: Chemical structure of folates. Image from Garratt et al. 2005. Folinic acid is 5/10 formyl-THF (tetrahydrofolate).

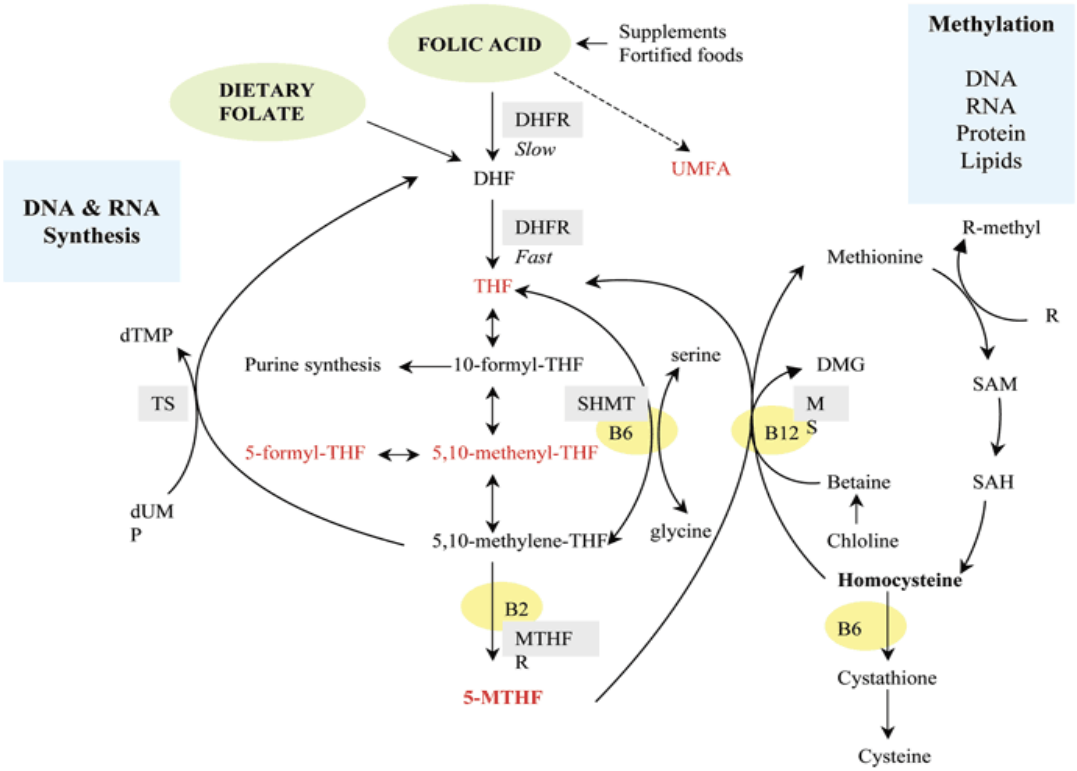


Figure 3: The Folate cycle Image adapted from Garratt et al 2005. Abbreviations: DHF = dihydrofolate, DHFR = dihydrofolate reductase, DMG = dimethylglycine dTMP = Thymidine monophosphate, dUMP = deoxyuridine monophosphate, MS = methionine synthase MTHF = methylenetetrahydrofolate reductase SAH = s-adenosylhomocysteine, SAM = s-adenosylmethionine SHMT = serine hydroxymethyltransferase THF = tetrahydrofolic acid, TS = Thymidine synthase UMFA = unmodified folic acid.

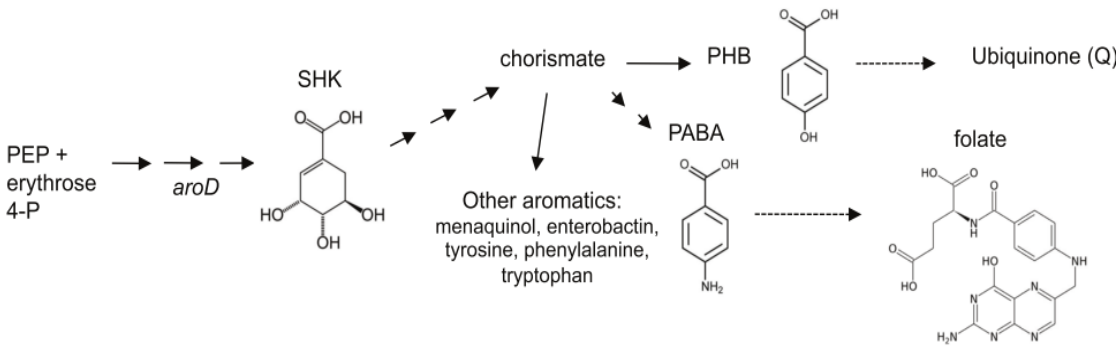


Figure 4: The shikimic acid pathway. Abbreviations: P = phosphate, PABA = para-aminobenzoic acid, PHB = para-hydroxybenzoic acid, PEP = phosphoenolpyruvate, SHK = shikimate,

Shane & Stokstad 1985; Reed et al. 2006; Lu et al. 2007). Folate species can act to either accept or donate one carbon units in several reactions collectively known as folate mediated one-carbon metabolism (Shane & Stokstad 1985). Tetrahydrofolate (THF) is the active form of folate coenzyme that is able to act as the donor or acceptor of one-carbon groups for key metabolic reactions, for example 10-formyl-THF is used in the biosynthesis of the purine ring (Shane & Stokstad 1985; Bailey & Gregory 1999). 5-10 methyleneTHF can be reduced to 5-methylTHF by methylene tetrahydrofolate reductase (MTHFR). This N-5 methyl group from 5-methylTHF is then used in the methionine synthase reaction which results in the synthesis of methionine from homocysteine. Methionine is required during protein synthesis, and also acts as a methyl donor via its conversion to S-adenosylmethionine (SAM) a primary methylating agent involved in over 100 methyltransferase reactions (Shane & Stokstad 1985; Bailey & Gregory 1999) (Figure 3).

All folate species have a varying number of glutamate molecules attached. The number of glutamates can vary from a single glutamate molecule, through to a chain of 9 glutamates attached to the folate species (Lu et al. 2007; Garratt et al. 2005; Stover & Field 2011). It is thought that polyglutamated folate species are the most common form of intracellular folates, and are the natural substrates for one-carbon metabolism enzymes. As substrates for these enzymes, they have shown to be as effective, with some more effective than monoglutamates varieties (Shane & Stokstad 1985). Polyglutamated species of folate may also be important in the regulation of folate mediated one carbon metabolism. There is evidence to show that polyglutamates varieties of folates effectively inhibit some of the enzymes required for one-carbon metabolism, whilst the monoglutamated forms of the same folate

species are weak inhibitors in comparison, suggesting that the glutamate polypeptide increases the affinity of the folate to certain enzymes (Shane & Stokstad 1985; Stover & Field 2011).

There is evidence to suggest that polyglutamated folates are not transported across the cell membrane, or if they are only poorly transported. This results in the metabolism of monoglutamated forms of folates to polyglutamated forms of folate within the cell. Meaning that cells are able to concentrate folate species to a much higher level than available in the external environment, and that the polyglutamates forms are more easily retained in the cell (Shane & Stokstad 1985; Stover & Field 2011).

1.10 Folate Deficiency

The folate cycle is highly complex, meaning that slight perturbations to dietary folate may ultimately lead to changes in folate status and risk of disease (Reed et al. 2006). Deficiency of folate has been shown to lead to a variety of disorders such as growth retardation, asthma, cardiovascular disease, cancer, neural tube defects in pregnant women and neurological disorders (McKay 2004; Kim & Yang 2004; Reed et al. 2006). Due to the substantial amount of evidence with regards to folate deficiency and neural tube defects during pregnancy, the folic acid fortification of flour was increased, and folic acid supplemented cereal-based products were made mandatory in the US and Canada in the late 1990's (Kim & Yang 2004).

1.11 *C. elegans:* a model organism

The free living nematode *C. elegans* has long been used as an excellent model organism for ageing research. In the wild, *C. elegans* are often found to proliferate on

rotting fruit or compost (Barrière & Félix 2005; Félix & Braendle 2010), however in the laboratory environment they can be maintained in petri dishes or in liquid culture, and are usually fed on lawns of the *E. coli* bacterial strain OP50 (Brenner 1974). *C. elegans* has a short generation time; the development from egg to egg laying adult takes around 3 days at 25°C. Fully developed adult *C. elegans* are approximately 1.2 mm in size, meaning that large numbers of animals can be grown efficiently and easily on petri dishes (Byerly et al. 1976).

1.11.1 *C. elegans* anatomy and lifecycle

An adult hermaphrodite is compromised of only 959 somatic cells, with the complete cell lineage, which is invariant between animals, having been established (Torok-Storb 1988; Herndon et al. 2002). Worms are enclosed by a cuticle, which is a sturdy yet flexible exoskeleton like structure to which the muscles are attached. Food is taken in through the mouth, compacted and pumped into the intestine by the pharynx. The intestinal lumen of the worm runs through the entirety of the body from the pharynx to the anus (Figure 5) (Ward & Thomson 1975; Francis & Waterston 1991).

The lifecycle of *C. elegans* starts with an embryonic stage, followed by four larval stages (named L1-4) followed by adulthood (Byerly et al. 1976). This lifecycle is observed in favorable conditions, where food is not limited and the population is not at such a high density that overcrowding occurs. If conditions are not favorable, if food is scarce, the population is too large or the temperature too high, then the worm is able to form a dauer. During dauer formation the larvae complete L1 developmental stage, but then go to a predauer stage known as L2d.Worms can remain in dauer state for up to 4 months, as they have high fat stores, are non feeding and highly resistant to

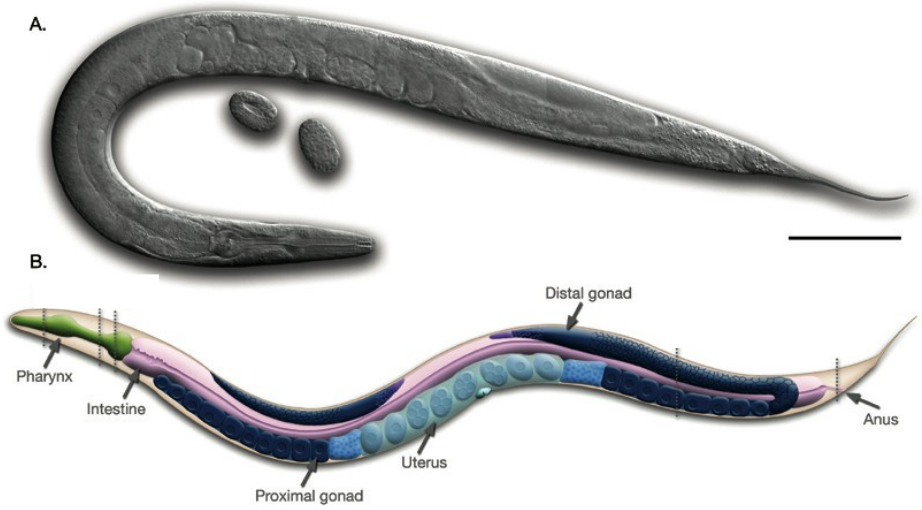


Figure 5: Anatomy of an adult hermaphrodite (Image from Worm atlas). **A.** DIC (differential interference contrast) image of an adult hermaphrodite, Scale bar 0.1 mm. **B.** Schematic drawing of anatomical structures, left lateral side.

stress. When conditions become favorable, dauers are able to re-enter the lifecycle at L4 stage (Figure 6) and progress through to adulthood as normal (Byerly et al. 1976). As mentioned, developmental time from egg to gravid egg laying adult takes 3 days at 25°C. Hermaphrodites finish their reproductive and egg laying cycle around day 6-7 or adulthood, with a typical total brood size of 300-400 eggs at 25°C. Typical adult lifespan of hermaphrodites is approximately 12-14 days at 25°C (Kenyon et al. 1993)

1.11.2 *C. elegans* as a genetic model organism

C. elegans have two sexes determined by the presence or absence of a second X chromosome. Males have only one copy of the X chromosome (XO), whereas hermaphrodites possess two copies (XX) (Brenner 1974). Hermaphrodites can either self fertilise or mate with males, but cannot mate with other hermaphrodites. If hermaphrodites are left to self fertilise, then males are relatively rare, approximately 1 in 1000 individuals will be male. However if hermaphrodites mate with a male, then progeny will be 50:50 male to hermaphrodites, as half of the progeny will not receive a copy of the X chromosome from sperm. Male animals are generally smaller than hermaphrodites, but can more readily be distinguished from hermaphrodites by the hook on their tail (Brenner 1974).

The self fertilizing trait of hermaphrodites is particularly useful in studies requiring isogenic populations, such as genetic screens or lifespan experiments. However, males are also useful for outcrossing with other worm strains, for example, to make a wild type sibling of an animal with a genetic mutation, or to make a double mutant (Brenner 1974).

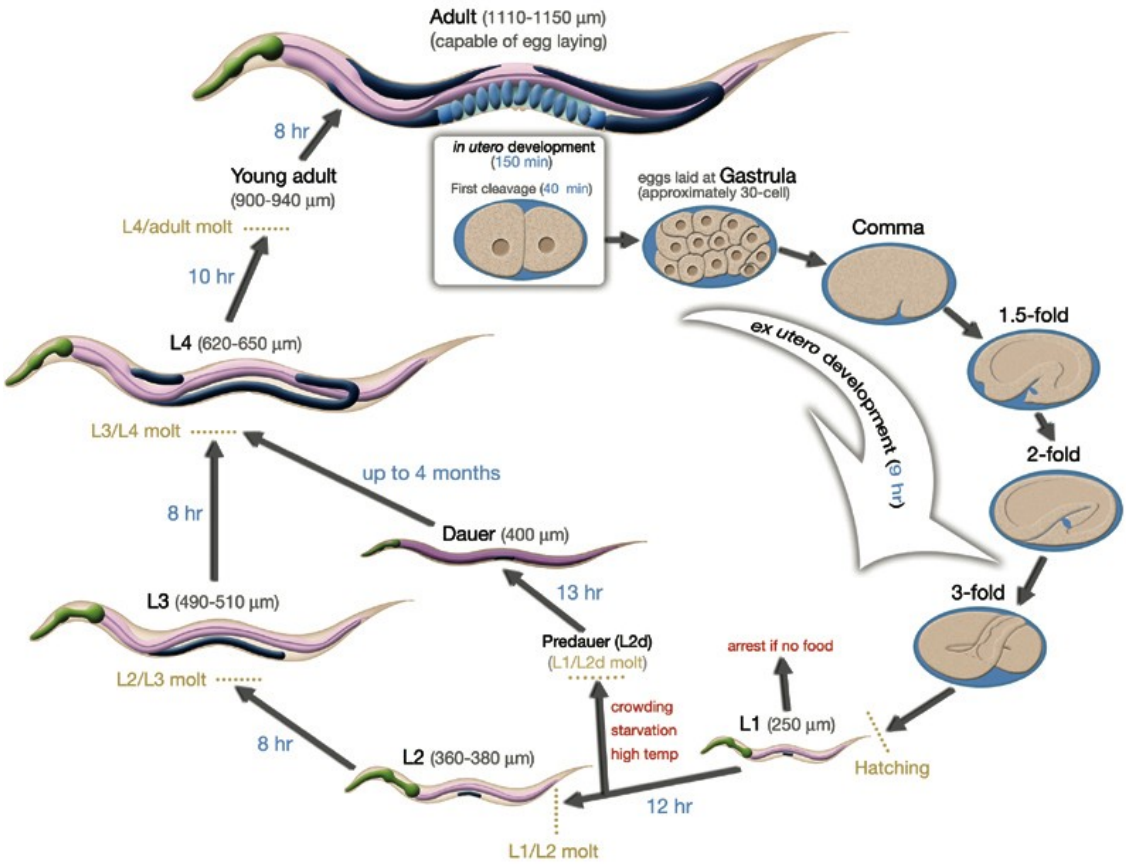


Figure 6: Lifecycle of *C. elegans* at 22°C. Life cycle from egg through four larval stages (L1-L4) to adulthood.

The *C. elegans* genome is predicted to contain 19,000 genes and was fully sequenced by the *C. elegans* sequence consortium which was first published in 1998 and can easily be manipulated (Reboul et al. 2001; Kamath & Ahringer 2003). Mutations can be induced using a wide range of mutagens – for example X-ray radiation. This makes *C. elegans* the ideal system in which to investigate genes responsible for a particular trait. A large number of verified genetic mutants are now readily available from the *Caenorhabditis* Genetics Centre (CGC). Strains of *C. elegans* can be stored for a number of years at -70°C, which is especially useful when working with novel or weak strains, as an aliquot of the original strain can be thawed if needed (Brenner 1974).

1.11.3 *C. elegans* as a model to study ageing

The *C. elegans* genome contains homologs of approximately two thirds of all human disease genes, and therefore the worm has become a popular organism for modeling human diseases including age-related diseases (Sonnhammer & Durbin 1997; Baumeister & Ge 2002). Initially research in the *C. elegans* aging field was focused on the genetics of ageing and single gene mutations that drastically increased lifespan of the animal, such as *age-1* and *daf-2*.

An early screen for long-lived mutants (Klass, 1983) revealed at least one long-lived strain, which was later found to contain a mutation in the gene *age-1*. *age-1(hx546)* is a recessive mutant allele in *C. elegans* that was found to result in a significant and robust lifespan increase in both male and hermaphrodite worms (Friedman & Johnson 2002). *age-1*(*hx546*) was found to be associated with a 75% decrease in hermaphrodite self-fertility. The study showed that the long-life and reduced-fertility

phenotypes cosegregate and are tightly linked to *fer-15*, a locus on linkage group II. *age-1*(*hx546*) did not affect the timing of larval molts, the length of embryogenesis, food uptake, movement, or behaviour when tested (Friedman & Johnson 2002). At the time, the mutant in *age-1* was a novel instance of a well-characterized genetic locus of which the mutant form results in increased lifespan.

By chance it was discovered that a mutant of the gene *daf-2* was also long-lived (Kenyon et al., 1993), and that this same mutant had already been shown to cause dauer formation at restrictive temperatures (the Daf-c phenotype). Following this finding, more Daf-c mutants were tested, but not all showed an extended lifespan. In three different alleles of the *daf-2* gene, highly significant effect on lifespan was found. Testing of other strains showing the Daf-c identified an allele of *daf-23* which extended lifespan significantly (Larsen et al 1995), but following further research it was discovered that this allele was in the same gene previously described as *age-1* (Malone et al 1996; Morris et al 1996). Due to work on dauer development, it had already been shown that the Daf-c phenotype of *daf-2* alleles required the presence of the *daf-16* gene, which has a Daf-d phenotype (Daf-d denotes that the strain is less capable of forming dauers as compared to wild type strains) (Riddle et al., 1981). This result led to the finding that the longevity phenotype of the *daf-2* mutant *C. elegans* also requires *daf-16* (Kenyon et al., 1993). Cloning and sequence analysis of the *daf-16* gene revealed *daf-16* to be a homolog of human fork-head transcription factors FOXO3 and FOXO4 (Ogg et al., 1997). This research led to the discovery that single genes in the insulin/IGF-1 signaling (IIS) pathway can have dramatic effects on *C. elegans* lifespan.

The discovery that *age-1* codes for a phosphoinositide-3-OH kinase (PI3K) (Morris et al., 1996) led to the identification of further downstream elements in the IIS pathway. Akt/PKB serine/threonine kinases had already been shown to act as downstream effectors of PI3 kinases in other model systems (Toker and Cantley, 1997). The newly sequenced *C. elegans* genome was used in attempt to identify homologs in worms. The two genes *akt-1* and *akt-2* were identified and found to have a role in signaling to *daf-16*. The activating mutation *mg144* in *akt-1* was capable of fully suppressing the Daf-c phenotype of *age-1* mutant *C. elegans.* Inhibition of both *akt-1* and *akt-2* activity led to complete dauer arrest. This Daf-c phenotype could be completely suppressed by a null mutation in *daf-16* showing that the two *C. elegans* Akt.PKB homologs AKT-1 and AKT-2 act redundantly to antagonize DAF-16 (Paradis and Ruvkun 1998).

There has been much research on the IIS pathway in *C. elegans*, and there is substantial evidence that the IIS pathway is an evolutionarily conserved mechanism of longevity from yeast to humans (Barbieri & Bonafè 2003). Similar to *daf-2* mutation in *C. elegans*, mutations in the insulin receptor homolog InR in *Drosophila melanogaster* also show a large extension in lifespan (Tatar et al 2001). Foxo, the *Drosophila* homolog of *daf-16* has also been implicated in longevity (Giannakou et al 2004). In mice, inhibition of the IIS pathway has been shown to have protective properties against age-onset neurodegeneration-linked proteotoxicity (El-Ami et al. 2014). In humans there is evidence to suggest that genetic variation within the FOXO3A gene is strongly associated with human longevity (Willcox et al. 2008).

More recently, many different approaches are being used in the *C. elegans* ageing field as well as genetic manipulations that influence lifespan, such as environmental manipulations for example hormetic treatments, evolutionary studies, population studies, models of age-related diseases and drug screening for life extending compounds (Olsen et al. 2006).

1.12 The *C. elegans/ E. coli* model system

The genetic manipulation of *E. coli* has great potential as a method of precisely manipulating the *C. elegans* diet to further investigate diet/longevity interactions. It is possible to grow *C. elegans* axenically (with no microbial food source), however this has detrimental effects on their growth and development (Lenaerts et al. 2008). There have been many studies researching and defining the most suitable axenic media for worms in order to provide further information on any effect on lifespan that the bacteria might be exerting on the animal. Despite these efforts, worms grown on these defined axenic media, do not grow or develop as well as worms reared on live *E. coli.*

Studies have investigated the effect of maintaining *C. elegans* on dead *E. coli* cultures. Worms raised on killed bacteria exhibit development problems. Growth arrested *E. coli* used as a food source for *C. elegans* rescues the effects of axenic culture (Lenaerts et al. 2008). With regard to this finding, further work has attempted to identify the component of *E. coli* that is necessary and required for normal *C. elegans* nutrition. Whilst one particular bacterial component which results in normal development is yet to be discovered, this research provides compelling evidence that for healthy growth and development of *C. elegans,* there is a nutritional requirement that is fulfilled by live, metabolically active microbes (Lenaerts et al. 2008).

My research uses the simplified *C. elegans* / *E. coli* model to investigate the intricate relationship between diet and ageing. In laboratory studies, *C. elegans* are fed monoxenically on *E. coli*, meaning that the worms are being fed a homogenous and genetically well characterized diet. *C. elegans* are reared and maintained in a controlled environment, resulting in negligible environmental change between individuals. For lifespan analysis, hermaphrodites are allowed to self fertilise in order to produce isogenic populations.

1.12.1 Interactions between *E. coli* and *C. elegans*

It has previously been reported that when *C. elegans* are maintained on non-proliferating bacteria, then they show an increased mean lifespan (Garigan et al. 2002; Walker & Houthoofd 2005; Saiki et al. 2008). The GD1 *E. coli* mutant has a deletion in the *ubiG* gene. *ubiG* codes for a methyl-transferase in the ubiquinone (also called coenzyme Q, or Q) biosynthetic pathway, and thus cannot produce coenzyme Q (Saiki et al. 2008). Q is found in both eukaryotes and prokaryotes where its main role is within the respiratory electron transport chain. Research shows that when *C. elegans* are maintained on GD1 (Q-less) bacteria, they have increased lifespan. Further experimentation shows that the lifespan effect is not caused specifically by the lack of Q, but rather because the GD1 bacteria exhibits a defect in respiration (Saiki et al. 2008).

As mentioned previously, *C. elegans* maintained on killed bacteria have growth defects. In contrast, it has been shown that despite the slow development, worms maintained on UV-killed bacteria exhibit an increased lifespan (Gems & Riddle 2000),

perhaps as a form of dietary restriction. In concurrence with this finding, another study shows that worms kept on non-proliferating bacteria (bacteria that have been treated with antibiotics to prevent proliferation), also show an increase in longevity (Garigan et al. 2002).

In the lab *C. elegans* feed on a range of bacteria and yeasts, however little is known about their diet in their natural habitat. *C. elegans* sampled from the wild, regularly show signs of bacterial infection, such as bacterial films on the worm cuticle which would be considered unusual in lab maintained strains. Freshly isolates worms are also found with live bacterial and possibly fungal accumulation in their intestines, causing a constipation phenotype (Félix & Braendle 2010).

1.13 A serendipitous *aroD* mutant

Whilst performing lifespan analysis on *C. elegans* using RNA interference (RNAi) by feeding, an *E. coli* HT115(DE3) RNAi strain was found to increase lifespan of the long lived *daf-2* mutant by 30-50% (Virk et al. 2012). Further investigation showed that the mutant possessed a mutation in the *aroD* gene, which causes a disruption of the shikimic acid metabolic pathway (Figure 4). The shikimic acid pathway is responsible for the synthesis of aromatic compounds such as aromatic amino acids, quinones and para-aminobenzoic acid, a pre cursor of folic acid. Plasmid complementation was then performed to confirm that the increase in lifespan observed was due to the mutation in the *aroD* gene, and was successfully able to rescue the lifespan extension observed. An independent *aroD* mutation was ordered from the Keio collection to verify that the lifespan extension seen was not allele or strain specific (Virk et al. 2012)

The *aroD* gene codes for the enzyme 3-dehydroquinate dehydratase, an essential enzyme in the shikimic acid pathway that is responsible for the production of chorismate. In conjunction with this, when shikimic acid was supplemented to the media, the lifespan effect caused by the *aroD* mutant was no longer observed. The *aroD E. coli* is able to grow on peptone media used for lifespan analysis, therefore the media must be able to provide the essential aromatic compounds that are required for growth, or the appropriate pre cursors of these compounds. To determine whether the extended longevity observed was due to any of these compounds being limited in availability, compounds that have been shown to support the growth of *aro* mutants were supplemented to the media; these include the aromatic compounds, the folate precursor para-aminobenzoic acid (PABA) and the quinine pre cursor para-hydroxybenzoic acid (PHB). Of these compounds only one, PABA, eliminated the extension of lifespan completely.

This finding suggests that a decrease in bacterial folate synthesis in the *aroD* mutant is the major cause of the increased *C. elegans* lifespan observed. However, when folic acid was added to the *aroD* mutant the long lifespan effect was only partially rescued (Virk et al. 2012). As literature suggests that *E. coli* cannot take up folic acid itself (Brown et al. 1974), this evidence seems to suggest that the lifespan effect seen is not *E. coli* specific – i.e. not solely due to a reduction in bacterial folates – and that decreased levels of folate in the worm may also have an impact on the extension in longevity.

The mechanism behind the increased longevity seen in *C. elegans* fed on *aroD* remains unclear. The *aroD* mutant should in theory inhibit folate synthesis; however

the *E. coli* must still be able to synthesize low levels of folate for them to be viable. It is possible that there are traces of PABA or shikimic acid itself present in the media, which the mutant *E. coli* are then able to synthesize into folate. Measurements of folate levels in both the *aroD* mutant and the wild type *E. coli* would confirm the difference in levels of folate between the mutant and wild type. Measurement of folate levels in worms fed on either mutant or wild type *E. coli* is also required to provide knowledge on the effects of folate.

1.14 Aims and Objectives

I intend to investigate further into the mechanism via which the *aroD E. coli* mutant extends *C. elegans* lifespan. I aim to determine whether decreasing folate levels is the cause behind the increase in animal lifespan. If a decrease in folate levels is the cause of the lifespan effect, then I will aim to reproduce the lifespan extension seen with the *aroD* mutant using chemical interventions to decrease folate levels. I will also try to delve further into the mechanism via which a decrease in folate levels causes an increase in *C. elegans* lifespan, and endeavor to determine whether the effect is caused by a change to the *E. coli*, a change to the worm or an interaction between the two organisms**.**

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 *C. elegans* strains used

Strain	Genotype/Relevant Characteristics	Source/Origin
CB1392	*nuc-1(e1392)*	(Lyon et al. 2000)
N2	Wild type	(Brenner 1974)
SS104	*glp-4(bn2)*	(Beanan & Strome 1992)
RB1055	*gcp-2.1(ok1004)*	Caenorhabditis Genetics Centre (CGC)
UF208	Wild type	This study
UF209	*gcp-2.1(ok1004)*	This study
UF215	*gcp-2.1(ok1004) gqEx37[gcp-2.1,Pgbp-2::GFP,*	This study
VC959	*tag-330(ok1460) V/nT1[qIs51](IV;V)*	(Balamurugan et al. 2007)

UF209 was outcrossed from the RB1055 strain 3 times, UF208 was a wild type sibling from the same cross

2.2 *E. coli* strains used

Strain	Genotype/Relevant Characteristics	Source/Origin
OP50	OP50 *ura*	(Brenner 1974)
OP50 sulfonamide (Su) resistant	OP50 (R26)	This study, (Villarroel & Hedges 1983)
OP50-GFP	OP50 (pFPV25.1)	(Valdivia & Falkow 1996)
aroD mutant	HT115(DE3) *aroD717::IS1*	(Virk et al. 2012)
Keio collection wild type	BW25113	(Baba et al. 2006)
Keio collection *aroD*	BW25113 *aroD*	(Baba et al. 2006; Yamamoto 2009)

2.3 Solutions for *C. elegans* maintenance

M9 buffer (7.37g/l $Na_2HPO_4.2H_2O$ (≥99.0%, Sigma-Aldrich), 3.00g/l KH_2PO_4 (≥99.0%, Sigma-Aldrich), and 5.00g/l NaCl (≥99.0%, Sigma-Aldrich), to distilled water, stirred until dissolved. The solution was then divided into 250ml aliquots and autoclaved at 121°C for 20 minutes to sterilise.)

LB (Luria Bertolani broth) (10.0g/l Tryptone (95039, Sigma-Aldrich), 5.0g/l Yeast Extract (Y1625, Sigma-Aldrich), 10.0g/l NaCl, to distilled water and mixed thoroughly. The solution was then divided into 250ml aliquots and autoclaved to sterilise.)

2.4 Nematode Growth Media (NGM) Plate Preparation for *C. elegans* maintenance

These plates were prepared in advance and stored at 4°C until needed. NGM was prepared by adding 3.00g/l NaCl, 2.50g/l Peptone (70171, Sigma-Aldrich) or Tryptone (dependent on type of plate required) and 20.0g/l Agar (05038, Sigma-Aldrich) to distilled water and mixed vigorously. The media was then autoclaved at 121°C for 20 minutes to sterilise. Once the autoclave cycle had completed, the media was cooled to 65°C at which point, 1ml/l of Cholesterol (≥99.0%, Sigma-Aldrich)(cholesterol solution final concentration 5mg/ml dissolved in ethanol), 1ml/l of 1M $CaCl_2$ (≥96.0%, Sigma-Aldrich), 1ml/l of 1M $MgSO_4$ (≥99.5%, Sigma-Aldrich) and 25ml/l of 1M KH_2PO_4 (pH 6.0) were added aseptically in a safety flow cabinet. The media was then aliquoted into 60 mm plates, ensuring that 15ml was pipetted into each plate.

2.4.1 Stock Solutions of compounds to be added to NGM plates

100x stock solutions of compounds to be added to plates (e.g. folic acid, SMX) were prepared, and the appropriate volume added to the cooled agar before the plates were poured. SMX and folic acid were dissolved in distilled H_2O, with 4M NaOH (≥98.0%, Sigma-Aldrich) added to aid dissolving of the compound. folinic acid (96.220, Schircks) came in a salt form, and therefore was dissolved in distilled H_2O alone. para-aminobenzoic acid (PABA) (≥99.0%, Sigma-Aldrich) and para-hydroxybenzoic acid (PHB) (≥99.0%, Sigma-Aldrich) are dissolved in >99% ethanol. All compound solutions once made were filter sterilised using a 0.2µm filter.

2.4.2 Preparation of NGM compound plates

When compound plates were required (NGM plates plus for example sulfamethoxazole (SMX) (S7507, Sigma-Aldrich) or folic acid (16.203, Schircks), the media was prepared and autoclaved as above. Once cooled and after the $CaCl_2$, $MgSO_4$ and KH_2PO_4 (pH 6.0) had been added, the appropriate volume of 100x stock solution of the compound (section 2.4.1) was added before the media is poured into the plates.

2.4.3 Preparation of Minimal Media Plates

For the *aroD* mutant bacterial growth assays, minimal media plates were required. When preparing the solutions, filter sterilising was performed using a 0.2µm filter, and when autoclaved, run on a 20 minute cycle at 121°C.

The following sterile stock solutions were prepared:

Trace Element Solution

5.00g/l Ethylenediaminetetraacetic acid (EDTA), disodium salt (≥99.0%, Sigma-Aldrich), 0.50g/l $FeCl_3$(≥97.0%, Sigma-Aldrich), 0.05g/l ZnO (≥99.0%, Sigma-Aldrich), 0.01g/l $CuCl_2$ (≥99.0%, Sigma-Aldrich), 0.01g/l $CoCl_2.6H_2O$ (≥99.0%, Sigma-Aldrich), 0.01g/l H_3BO_3 (≥99.0%, Sigma-Aldrich) added to distilled water, pH of solution was then adjusted to pH 7.0. The solution was then autoclaved.

10% Glucose (G0350500, Sigma-Aldrich) solution filter sterilised.

20x Phosphates and $(NH_4)_2SO_4$ solution

86.92g/l Na_2HPO_4 anhydrous (≥99.0%, Sigma-Aldrich), 54.00g/l KH_2PO_4, 20.00g/l $(NH_4)_2SO_4$ (≥99.0%, Sigma-Aldrich) added to distilled water and then autoclaved.

Amino Acid Solution

40.0g/l of amino acid mix (see table below) added to distilled water and then autoclaved. All amino acids from Sigma-Aldrich

Adenine	0.8g
Arginine	0.4g
Asparagine	1.0g
Cysteine	0.8g
Glutamic Acid	1.0g
Histidine	0.8g
IsoLeucine	0.6g
Leucine	1.2g
Lysine	0.6g
Methionine	0.8g
Phenylalanine	0.6g
Serine	4.0g
Threonine	2.0g
Tryptophan	0.8g
Tyrosine	0.4g
Uracil	0.8g
Valine	1.5g
TOTAL	**18.1g**

10.00 g/l Ca $(NO_3)_2$ (≥99.0%, Sigma-Aldrich), filter sterilised.

1% Thiamine (≥99.0%, Sigma-Aldrich), filter sterilised

0.5M $MgSO_4$, filter sterilised

The above sterile stock solutions were combined (20X phosphates and $(NH_4)_2SO_4$ solutions (50ml), Amino Acid solution (50ml), 10% Glucose (20ml), Trace element

solutions (10ml), 0.5M Mg_2SO_4 solution (2ml), 10mg/ml $Ca(NO_3)_2$ (1ml), 1% Thiamine (0.5ml)), and the resulting solution (total volume 133.5ml) was heated in a water bath at 65°C and then added to 766.5ml agar solution (20g/l agar) that had been autoclaved and cooled. The solution was then aliquoted into 60mm plates, ensuring that each plate contained 15ml of media. The poured plates were then left to dry and stored at 4°C until needed.

2.5 Preparation of bacterial cultures

To prepare cultures that were then available to use for seeding plates, 35ml of LB was pipetted aseptically into a sterile 50ml polypropylene tube. A disposable inoculating loop was then used to inoculate the LB with *E. coli* from either a frozen stock culture or an existing culture if there was no contamination. Once inoculated the tubes were then placed into an incubating orbital shaker (Stuart SSL1 Lab-scale orbital shaker) at 37°C and set to 220rpm and allowed to grow overnight. The liquid cultures were then stored at 4°C, until required for seeding plates.

2.6 Measuring bacterial growth on minimal media plates

Bacterial growth assays were carried out using minimal media plates. If compounds were to be added to the minimal media, stock solutions were prepared and added to the set minimal media plates, then spread under sterile conditions and allowed to dry. Once the plates had dried, bacteria was streaked onto the entire plate using a disposable inoculating loop. The plates were then stored at 37°C overnight and checked the following day for growth. If the colonies found were small, the plates were stored at 37°C overnight for a second time, checked again the following day for growth, and images taken.

2.7 Seeding plates with *E. coli* as a food source for *C. elegans*

Plates must be seeded 2-3 days prior to usage. Plates were seeded with 100µl of bacterial culture (section 2.5) that was placed onto each agar plate using a repeat pipette under sterile conditions. The plates were then left on the bench to dry and kept at room temperature until use.

2.8 *C. elegans* strain maintenance

To ensure that animals were available for lifespan experiments and other research when required, populations of all cultures were kept and maintained on NGM plates. To keep the populations going, when the animals had starved, sections of the plate with starved worms on were cut and placed onto new plates. The cut out section was placed onto a seeded plate away from the edge of the bacterial lawn. The starved worms then move towards the *E. coli* food source, and continue development.

2.9 Obtaining a synchronised population of *C. elegans* by bleaching

This method was used to obtain eggs from gravid unstarved adult worms that were free from the *E. coli* they were grown on and any contamination. To ensure that enough eggs were obtained, generally 8 plates were used for bleaching, with 2 plates being kept aside for strain maintenance. In order to ensure that enough worms were produced for the egg lay stage, 2 plates per lifespan experiment being performed were prepared with eggs from the bleaching protocol.

Each plate was washed with 1-2ml of M9 solution, into a 1.5ml eppendorf tube. The M9 from 2 plates was washed into 1 eppendorf tube. The adult worms were allowed to

settle on the bottom of the tube, once this had occurred the excess M9 was removed, leaving approximately 200µl left in the tube which contained both the worms and M9 solution. Add 125µl of bleach solution (7:8 sodium hypochlorite (425044, Sigma-Aldrich) and 4M NaOH respectively) to each tube and the tubes were then shaken continually by hand for approximately 3 minutes, monitoring by eye the loss of worm definition and cloudiness of solution. Time taken for bleaching process to complete depended on lab temperature, age of bleach and number of worms. Once worm definition had been lost and the solution had become clear of worm carcasses, the tube was immediately filled with M9, and centrifuged for 1.5mins at 3000rpm in a Spectrafuge 24D bench top centrifuge (Jencons-PLS). After centrifugation, the supernatant was removed, leaving approximately 20-40µl in the tube as to not remove the pellet of eggs. To wash the eggs and remove traces of bleach, the tube was then refilled with M9 and the pellet dissociated by shaking. The tubes were then centrifuged again as before (1.5mins at 3000rpm in a Spectrafuge 24D), the supernatant removed, and the tube filled again with M9 for the final wash stage. After the third and final centrifugation (1.5 mins at 3000rpm in a Spectrafuge 24D), the supernatant was removed as before – leaving 20-40µl in the tube. The numbers of eggs obtained were then observed by eye under the microscope. To quantify the number of eggs more accurately, the eggs were resuspended in the solution left in the tube, and 5µl of this was then placed onto a seeded NGM plate. The number of eggs in 5µl was then calculated, and eggs placed onto the seeded plates appropriately – ensuring that there were 50-200 eggs per plate.

2.11 Obtaining a synchronised population of *C. elegans* by egg lay

An egg lay is performed to gain synchronised populations for experiments such as

lifespans. The worms produced from the bleaching protocol (section 2.9) cannot be used for lifespan experiments as the bleach may have had an adverse effect on them. Therefore, the next generation is used for lifespans. To make sure that there were enough worms for each lifespan experiment, 4-5 plates per lifespan were used for the egg lay.

For the egg lay, 3-5 gravid adult worms were placed onto seeded NGM plates (as previously mentioned if the lifespan experiments were comparing mutant to wild type these must be seeded with mutant *E. coli*), and placed at 15°C overnight. The following day, the plates were checked to ensure that eggs had been laid, and the 3-5 adult worms were removed. The eggs were then placed back into the 15°C incubator and allowed to develop to late L3 or early L4 stage.

2.11 Measuring the lifespan of *C. elegans*

Lifespan experiments were performed using SS104 strain *C. elegans*. This strain was used due its temperature sensitive sterility – at 25°C the adult animals are sterile – this made lifespan experiments less complicated as the worms did not need to be transferred daily to avoid confusion with progeny.

Sections of plates containing starved worms were cut and placed onto 8-10 seeded NGM plates (Figure 8).These plates were then kept in an incubator at 15°C for 4-5 days until the worms had become gravid, unstarved adults. The animals were then bleached to obtain synchronised populations (see section 2.9). Once the eggs had been obtained, they were placed onto seeded plates. If the lifespan being carried out was comparing the effect on *C. elegans* lifespan of mutant *E. coli* and wild type *E coli*, the

plates that the eggs were put on to were seeded (see section 2.7) with the mutant *E. coli* (or OP50 + the highest concentration of SMX if experiment was measuring effect of lifespan on OP50 compared to OP50 plus SMX). The plates with the eggs on were then placed back into the 15°C incubator for 4-5 days. Once the eggs had hatched and the animals had become gravid, an egg lay was performed (see section 2.10). After the egg lay, plates with eggs on were kept at 15°C for 3-4 days until the worms had developed to late L3 or early L4 developmental stage. At this point, the worms were shifted to 25°C to induce sterility and kept overnight at 25°C. The following day, when the worms were at L4 stage or young adults, they were picked. Twenty five worms were picked onto a seeded plate, with 5 plates per lifespan – a total of 125 worms per lifespan experiment. The animals were left on these plated for the first 7 days, after this all worms were transferred on to fresh seeded plates and the scoring of the lifespan began. Day 1 of scoring was regarded as the first day of adulthood (young adult developmental stage). The worms were scored as Alive, Dead or Censored, and scored every alternate day. After the first transfer at 7 days, the worms were again transferred onto new seeded NGM plates at day 14. After this transfer there were a small number of animals left and therefore no further transfers were required. If plates developed mild contamination, the area of agar with the contamination was removed, and any worms in the contaminated area scored as censored. If plates developed severe contamination, all worms on the plates were scored as censored.

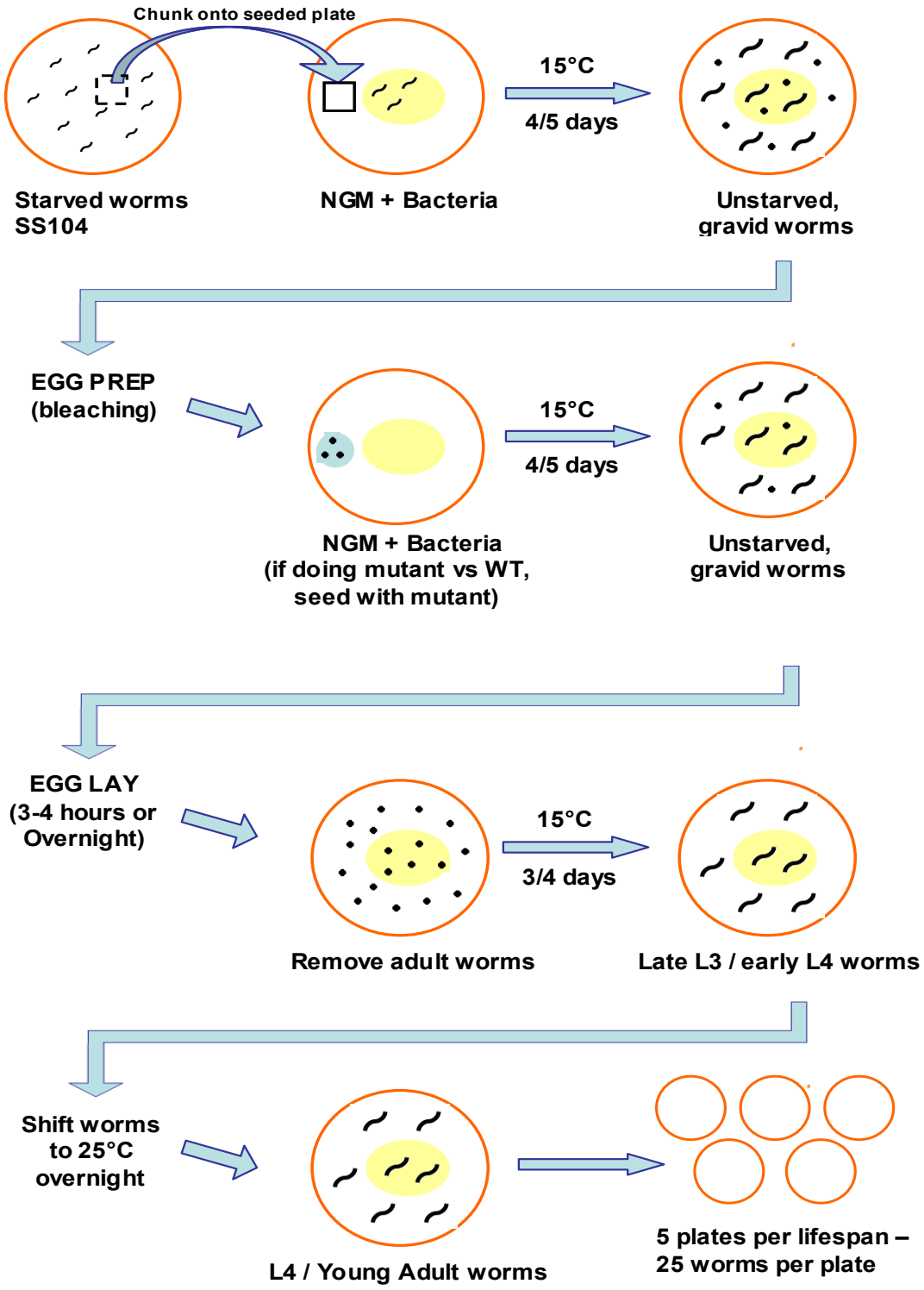


Figure 7: Schematic of lifespan protocol, adapted from Marjanne Bourgeois. Lines represent *C. elegans*, black dots represent *C. elegans* eggs, blue dot containing black dots represents *C. elegans* eggs from bleaching protocol.

2.12 Single Worm PCR

2.12.1 Preparation of Single worm lysis buffer

Single worm lysis buffer (50mM KCl (≥99.0%, Sigma-Aldrich), 2.5mM $MgCl_2$ (≥99.0%, Sigma-Aldrich), 10mM Tris HCl (pH 8.3) (RES3098T-B7, Sigma-Aldrich), 0.5% NP40 (NP40, Sigma), 0.5% Tween 20 (P1379, Sigma-Aldrich), 0.01% gelatine (G7041, Sigma-Aldrich) was prepared and stored at -20°C in 1.5ml aliquots.

10µl/ml Proteinase K (P2308, Sigma-Aldrich) must be added to the lysis buffer, before the worms were added.

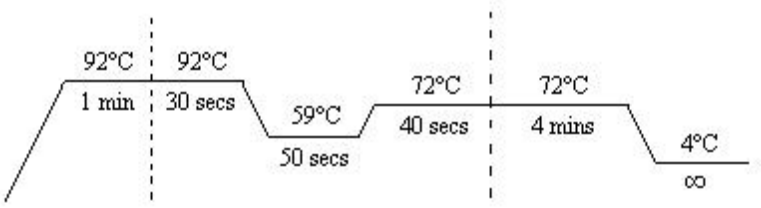
2.12.2 Single worm lysis

In order to obtain *C. elegans* DNA for use in PCR reactions, single worm lysis was performed. 5µl of single worm lysis buffer (see section 2.12.1) with added proteinase K was placed into each lysis tube. Once the buffer had been added, a single worm was picked and added to each tube containing lysis buffer. The tubes were then placed into a Thermocycler (Mastercycler 96 well/Thermocycler 5333, Eppendorf) programmed to run at 60°C for 60 minutes, and then hold the samples at 95°C for 15 minutes.

2.12.3 PCR reaction used for single worm PCR

When preparing the PCR reaction, PCR master mix was made – for 16 PCR reactions, enough master mix for 20 reactions was prepared (Promega PCR master mix (2x) (200µl), Folt-1 (7) primer (8µl), Folt-1 (8) primer (8µl), Folt-1 (9) primer (8µl), distilled water (136µl) – Total volume 360µl) 2µl of the DNA from single worm lysis was added to each PCR tube, and then 18µl of PCR master mix was added.

The tubes were then place in a Thermocycler (Mastercycler 96 well/Thermocycler 5333, Eppendorf) set for the following programme (35 cycles):



Upon completion of the PCR programme, the samples were run on a 1.8% agarose gel and visualised using ethidium bromide.

Single worm PCR methodology used was adapted from Plasterk lab protocols (Williams et al. 1992).

The primers used in the PCR reactions were as follows:

Folt-1 (7): 5' - CGA CCA GCT ACT CCA TTC CTC AC - 3'

Folt-1 (8): 5' – AGA ACC GAG CCT CTT GAA AC – 3'

Folt-1 (9): 5' – GCA AGA GCC CAC CAC AAT GAC C – 3'

Primers were designed using ApE - A Plasmid Editor Version 1.17 (M. Wayne Davis 2009).

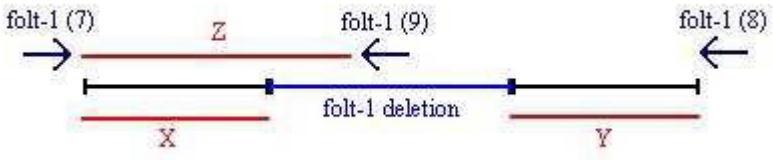


Figure 8: Positions of *folt-1* primers used for genotyping

Folt-1 PCR was performed with 3 primers (folt-1 (7),(8) and (9)). The expected PCR products were:

In folt-1 mutants that possessed the folt-1 deletion: X+Y = 646 bp

In folt-1 wild type animals that did not possess the folt-1 deletion: Z = 809bp

This allowed us to genotype worms as homozygous mutant, homozygous wild type or heterozygous, as the bands were different enough in size that they would be resolved when products were run on a gel.

2.12.4 Agarose gel electrophoresis

10 µl of each sample was run on a 1% agarose (MB1200, Melford) gel and visualised with ethidium bromide. Gels were run for approximately 30 minutes at 110V.

2.13 Detection and quantitative determination of *in vivo* folate by mass spectrometry

2.13.1 Solutions for in vivo folate extraction

Extraction buffer used was a 50:50 mix of aqueous Buffer (80:20 methanol: distilled water, 0.1% ascorbic acid (≥99.0%, Sigma-Aldrich), 20mM ammonium acetate (≥98.0%, Sigma-Aldrich)) and methanol. Extraction buffer was also spiked with methotrexate that had 6 glutamates (16.416, Schircks) (2 µl in 1000 µl), as methotrexate with 6 glutamates does not occur in nature, and therefore allowed us to ensure that the extraction protocol was efficient. This buffer was made fresh daily, and stored on ice before use. Methotrexate with 6 glutamates, 5-formyl tetrahydrofolate with 3 glutamates (16.283, Schircks), and folinic acid were used as standards at a concentration of 1mg/ml.

2.13.2 Folate extraction protocol for *E. coli* samples

8-10 plates were seeded with 100 µl of bacterial culture and left at room temperature for 2 days. After this the plates were moved to 25°C for 1 day, so as to emulate the conditions used during a lifespan experiment.

For extraction, approximately 1.5ml of M9 was placed on to each plate, and the *E. coli* lawn scraped with a glass spreader under sterile conditions. The M9 containing the *E. coli* from each of the plates, was then taken off and pooled into a 15ml polypropylene tube. The volume of contents in each tube was then measured, and the optical density at 600nm (OD_{600}) measured. Samples were diluted 1 in 5 (200µl of sample added to 800µl of M9) for OD measurement. The 15ml tubes were then placed in a centrifuge (Sanyo Harrier 18/80 refrigerated) and spun at 4000rpm for 5 minutes. The samples were then removed from the centrifuge and the supernatant poured away. The bacterial pellet in each tube was left to dry for 2 minutes, after which the tubes were placed in liquid nitrogen to snap freeze the bacterial pellet.

To calculate the volume of extraction buffer (see section 2.13.1) to be added to each sample, the volume of contents in each tube was multiplied by the OD_{600}. This value was then multiplied by 37.5 (an arbitrary value calculated from preliminary work) to obtain the volume of buffer to be added in µl. Using this method the volume of buffer per bacteria would be constant over all samples. After 10 minutes the tubes were removed from the liquid nitrogen and the calculated volume of extraction buffer, added to each tube. As the pellet was frozen, vigorous mixing was required to mix the bacterial pellet with the extraction buffer. Once mixed thoroughly, each sample was then transferred into a sterile eppendorf tube, and then sonicated in an ice bath for 10

seconds, 3 times with a pause of 20 seconds in between each sonication to prevent the probe from heating.

The samples were then centrifuged (eppendorf centrifuge 5415 R) further at 4°C for 5 minutes at 13000rpm. The supernatant was transferred to a sterile eppendorf, as the supernatant contained the folates that have been extracted. At this stage the samples were either stored at -80°C, and centrifuged again before they were to be run; or if they were to be analysed on the same day as preparation, spun in the eppendorf centrifuge 5415 R at 13000rpm for 5 minutes at 4°C. After this final centrifugation, the supernatant was again transferred to a sterile eppendorf tube and taken for analysis.

2.13.3 Folate extraction protocol for *C. elegans* samples

The extraction protocol used for worm folates was similar to the bacterial extraction, however there were a few adaptations. One such change was that the samples were snap frozen with quick thawing twice to break open the worm cuticle, not once as in the bacterial samples. Also instead of adding extraction buffer to the pellet after snap freezing, methanol spiked with methotrexate 6 glutamates was added. Instead of sonication, glass beads were added to each sample. The samples were covered in foil and securely placed on to an orbital shaker set to 125rpm (Stuart SSL1 Lab-scale orbital shaker) at 37°C for 1 hour.

2.13.4 Analysis of folate levels in *E. coli* and *C. elegans* extraction samples

The protocol for folate extraction and running of samples was obtained by adapting procedures from Garratt *et al* (2005) and Lu *et al* (2007). Samples were run using a C18 reversed phase column (Waters Acquity BEH, 100 mm x 2.1 mm) reverse phase

high pressure liquid chromatography (HPLC) with dimethylhexylamine (98%, Sigma-Aldrich) as an ion pairing agent. The mobile phase consisted of methanol with 5mM dimethylhexylamine, at a flow rate of 0.2mL/min. The injection volume was 10 µl. The mass analysis was run by negative electrospray ionisation time-of-flight (negative ESI TOF) utilising a quadrupole time-of-flight (Q-TOF) Premier instrument (Waters Corporation) calibrated with sodium formate and with dynamic correction from a leucine encephalin lock spray. Sampling cone voltage was -35V and capillary voltage was -2500V. The peaks obtained by selecting the specific mass were integrated using MassLynx software (Waters Corporation) and used as an indication of quantity. The peaks generated by known metabolites were also integrated and the peak corresponding to coenzyme A was chosen for use in normalization because it was large and there was little variation between samples.

2.14 *C. elegans* fecundity assay

All fecundity assays were performed using N2 strain *C. elegans* at 25°C so as to emulate the conditions experienced during lifespan experiments. Populations of N2 worms were synchronised by bleaching (see section 2.9) and the eggs obtained pipetted onto NGM plates supplemented with 128µg/ml SMX. Once hatched, the larvae were allowed to develop to gravid adults and then an overnight egg lay (see section 2.10) on 128µg/ml SMX plates was carried out. After the egg lay, plates were kept for 3 days or until the worms had developed to early L4 developmental stage. Individual L4 worms (n=20) were picked onto 20 separate plates per condition. In this case there were 3 conditions: control (no supplementation), 16 µg/ml SMX and 128 µg/ml SMX, and this was counted as day 0 of adulthood. Once worms had begun laying eggs they were transferred to freshly seeded plates daily. The previous plates

were kept at 25°C for 1-2 days to allow the larvae to develop and then the number of viable progeny was counted. Dead eggs were not included in counts of progeny, and counts of progeny of worms that then crawled off of the plate were not included in final analysis.

2.15 *C. elegans* motility assay

Motility assays were performed using SS104 strain worms. Bleaching (section 2.9) and an egg lay (section 2.10) were carried out as described above with worms being grown and developing on plates supplemented with 128 µg/ml SMX and maintained at 15°C. Once progeny from the egg lay had developed to L3/early L4 stage the plates were moved to 25°C to induce the temperature sensitive sterility associated with the SS104 strain. The next day, worms that were at L4 stage were picked - 10 worms per plate were put onto 16 plates per condition (n=160 per condition). In this case there were 3 conditions: control (no supplementation), 16 µg/ml SMX and 128 µg/ml SMX, and this was counted as day 0 of adulthood. Motility was scored from day 1 every alternate day until all worms were dead. Motility was scored as one of 3 classes A, B, C or dead (Herndon et al. 2002)

2.16 Transformation of plasmid R26 from *E. coli* C600 into *E. coli* OP50

(Method from John Ward, UCL, April 2011)

Two sets of plates were prepared; NGM plates as above and minimal M9 plates. Minimal M9 plates were prepared by adding 6.00 g/l Na_2HPO_4, 3.00 g/l KH_2PO_4 and 5.00 g/l NaCl and 20.0 g/l agar, then autoclaving for 20 minutes at 121°C. Once the media had been autoclaved, 10 ml/l of 2M NH_4Cl (≥99.0%, Sigma-Aldrich), a final

concentration of 4µg/ml of Uracil (≥99.0%, Sigma-Aldrich), 1ml/l of $MgSO_4$, 1ml/l of $CaCl_2$, a final concentration of 0.4% Glucose and 32µg/ml SMX were added to the media before the plates were poured. This concentration of SMX was used as it is selective against OP50. The two *E. coli* strains were grown separately in LB for OP50 and LB with 50 µg/ml ampicillin for C600 R26. Then 50µl of overnight culture of each strain was taken, and pipetted onto the same spot of a NGM plate (both strains are now mixed). Using a sterile spreader, the cells were spread out onto the NGM plate, and grown overnight at 37°C. The next day a swath of growth was removed from the NGM plate and streaked out onto a minimal M9 plate containing SMX to obtain single colonies. This plate was then incubated at 37 °C for 48 hours to allow single discrete colonies to grow. Minimal M9 plates were prepared as above, however in place of SMX, a final concentration of 50 µg/ml ampicillin (A1593, Sigma-Aldrich) was added. Onto these plates single individual colonies from the minimal M9 plate containing SMX were streaked out, one colony per plate. If the colonies from the M9 SMX plates grew on the M9 ampicillin plates then R26 plasmid had been successfully transferred. Permanent stock cultures of this *E. coli* OP50 R26 strain were then made and the strain was also frozen for future use.

2.17 Measuring the GFP Intensity of OP50-GFP

E. coli were seeded (see section 2.7) onto plates with the appropriate supplementation and left at room temperature for 2 days. After this time, the plates were placed at 25°C overnight. Images of the middle of the lawn were taken using a Leica M165 FL stereomicroscope with a GFP2 filter. Images were analyzed in ImageJ. Files were opened, and equivalent areas of each image measured for intensity.

2.18 Measuring the OP50-GFP accumulation within the intestinal lumen of *C. elegans*

Worms were prepared for and set up for lifespan analysis as described (see section 2.11); using GFP OP50 to seed plates (see section 2.7). All worms were checked daily for *E. coli* accumulation from day 1 of adulthood using a Leica M165 FL stereomicroscope with a GFP2 filter that has a 510 nm + long pass emission spectrum that allows GFP to be distinguished from autofluorescence. Once animals begun to accumulate *E. coli*, they were scored for both survival and accumulation every 2 days. Individual worms were scored as having no accumulation, partial accumulation or full accumulation. For this study only accumulation in the lumen of the worm was included, with bright spots in the pharynx area or elsewhere discounted.

2.19 Production of the *gcp-2.1* genomic transgene

To make the *gcp-2.1* genomic transgene, a genomic fragment containing the predicted *gcp-2.1* gene was amplified using the primers

R57gen_5: 5'CTTAGGTTGGATCTCGTTGCTTGC 3' and

R57gen_3: 5' TGTGTGGAAAGTGTGGTGAAGC 3'

using N2 genomic DNA as a template. 10 ng/µl of the PCR fragment with 90 ng/µl of marker plasmid *gpb-2*::*GFP* (Van der Linden 2001) was injected into UF209 *gcp-2.1(ok1004)* worms using a Leica DMI6000B microscope and Eppendorf FemtoJet and InjectMan Ni2 microinjection unit. A line transmitting the transgene mosaically, as assessed by GFP, was isolated (UF215).

2.20 Statistical Analysis

Analysis of lifespan data was performed using statistical software JMP Version 8.0.2 (SAS Institute Inc 2009). JMP analyzes lifespan data by calculating estimates of survival functions using the product-limit Kaplan-Meier model for one or more groups of right-censored data. Statistical significance is then determined using Log-Rank and Wilcoxon tests of fitting to the Kaplan-Meier model.

CHAPTER 3

BOTH *aroD* AND a TARGETTED CHEMICAL INTERVENTION INCREASE LIFESPAN IN *C. ELEGANS*

3.1 Introduction

The *aroD* mutant *E. coli* provides us with a novel potential life extending pathway to investigate. With its substantial increase in animal longevity, it is clear that it has benefits for the animal. The exact pathway or mechanism behind this extended lifespan is not clear. Further investigation is needed to determine whether this increase in longevity is associated with any negative side effects such as reduced fertility. Uncovering the mechanism via which the *aroD* mutant is working is essential, as mutating gut bacteria is not a viable option for the human population.

As described in chapter 1 (section 1.13), data suggests that *aroD* may be causing its effect via a folate dependent pathway, rather than other aromatics. The novel *aroD* mutant was found in a HT115(DE3) strain of *E. coli*, commonly used for RNAi (Timmons et al. 2001). In order to investigate the role of the folate pathway with regards to ageing in other strains of *E. coli*, including the *E. coli* strain most commonly used in *C. elegans* research, we may be able to use a drug to mimic the effect of the *aroD* mutant *E. coli* it is having. If successful, this drug has the potential to be taken as a supplement, a more feasible intervention for humans. The sulfonamides, also known as anti-folate drugs would be the ideal class of drug to investigate for such a drug.

3.1.1 Sulfa drugs

Sulfa drugs, which are also known as sulfonamides, represent some of the world's most widely used antibiotics, and are known to inhibit folate synthesis (Connor 1998; Castelli 2001). They are structurally analogous to para-aminobenzoic acid (PABA), in that sulfonamides have the substitution of the carboxyl group in PABA with a sulfa derivative. (Castelli 2001). During folate synthesis, PABA and dihydropteroate pyrophosphate condense to form dihydropteroate with the aid of the enzyme dihydropteroate synthase (DHPS). The dihydropteroate is then used as a substrate to form dihydrofolate (DHF) and ultimately tetrahydrofolate (THF) (Castelli 2001). Sulfonamides compete with PABA for DHPS, with some sulfonamides reported to be alternative substrates for the DHPS enzyme, which can convert sulfonamides to sulfa-derivative. Due to this competition, sulfonamides inhibit folate synthesis, resulting in folate depletion (Roland et al. 1979; Castelli 2001). Animals and humans cannot synthesis folates, as they lack the required enzymes. Due to the lack of DHPS in particular, sulfonamides can be used effectively in humans.

In 1932 it was reported that a red dye 4'-sulfamyl-2,4-diaminoazobenzene, more commonly known as prontosil, showed a consistent curative action for streptococcal infection in many experiments (Domagk 1957; Stokstad & Jukes 1987). Shortly after this it was announced that prontosil and other sulfonamide related compounds also proved effective against pneumococcal and staphylococcal infections (Domagk 1957). In 1935 these findings were published, making prontosil the first sulfonamide to be accepted as having a practical value with regards to treating infectious disease (Domagk 1957). The publication of these data led to intensive research in this field in several countries. With the interest in sulfonamides growing, treatments for many

diseases such as gonorrhea and meningitis epidemica became available and were very successful – after a few years of sulfonamide treatment the number of children's lives saved was approximately 10,000 in England alone. (Domagk 1957). Sulfonamides enabled the causal treatment of many diseases for the first time, especially when combined with other antibiotics, which were introduced later, such as penicillin. More recently sulfonamides were investigated for being used to treat malaria, as they inhibit malaria parasites (Ferone 1977), and are currently being looked into for treatment of HIV due to their anti-viral properties (Scozzafava et al. 2003). One sulfonamide in particular, indisulam (E7070), is in advanced clinical trials in Europe and the USA as a new anti-cancer agent, it treats solid tumors via a multifactorial mode of action. Indisulam has been found to strongly inhibits carbonic anhydrase, a critical enzyme shown to be associated with cancer involved in many physiological processes, and significantly alters gene expression levels of several transcripts (Supuran 2003).

There are many different sulfonamide drugs, and in the 1970's and 1980's in particular, different sulfonamides were used widely to treat different diseases such as urinary tract infections (UTI's), acute respiratory tract infections, and Crohn's disease (Björkman & Phillips-Howard 1991). However, as successful as these drugs were, they were not without reported side effects. Side effects reported ranged from mild to fatal, and several organs and tissues were reported to have been effected, with reactions related to blood, skin or liver being reported for all sulphonamides (Björkman & Phillips-Howard 1991), it is worth mentioning however that doses of drugs used when these side effects were observed were high (Björkman & Phillips-Howard 1991).

In this research sulfamethoxazole (SMX) was used, as it is still used clinically to treat urinary tract infections in combination with trimethoprim, Trimethoprim makes the bacteria more susceptible to SMX (Dodd 2004). The SMX-trimethoprim combination is administered at a much lower dose 480 mg single strength (80 mg trimethoprim: 400 mg SMX), or 960 mg double strength (160 mg trimethoprim: 800 mg SMX) per day administered three times a week, compared to the Björkman study mentioned above, where the dose of SMX used was 1600mg (Fischi et al. 1988; Ruskin & LaRiviere 1991; Leoung et al. 2001).

3.1.2 Preliminary study on SMX for use in this study

Preliminary work performed by a volunteer in the lab Andrea Bender and David Weinkove in our lab set out to determine concentrations of SMX supplementation that could be used in nematode growth media (NGM) plates, that would still allow the growth of the OP50 *E. coli* used. A range of concentrations were tested from 8 to 512 µg/ml final concentration SMX to determine the concentration that would prevent *E. coli* growth. At all of these concentrations there was a visible *E. coli* lawn, therefore these doses of supplementation were able to treat the *E. coli* and still allow growth. After these initial tests, lifespan analysis was performed using SMX supplementation, with data showing that worms maintained on SMX-treated *E. coli* having extended lifespan. However, Andrea did not have previous experience of working with *C. elegans*, and had not performed lifespan analysis before. In addition to this, the numbers of animals in these lifespans were small so we could not be confident in the results obtained.

3.1.3 Aims and Objectives

The novel *aroD* mutant *E. coli* had been shown to cause a significant increase in *C. elegans* lifespan. The *aroD* gene is involved in the early stages of the shikimic acid pathway, meaning it is upstream of the resulting folates produced, and thus there are several steps before the final folate product in which the mutant could be causing the lifespan extension. In this chapter, firstly I aim to test whether animals fed on the *aroD* mutant *E. coli* are indeed long lived in the lab conditions in Durham. Secondly, I aim to use SMX as a targeted pharmalogical intervention to mimic the effect of the novel *aroD* mutant on worm lifespan in OP50 *E. coli* widely used in *C. elegans* research, and determine whether the lifespan extension observed is indeed folate specific

3.2 Results

3.2.1 Replicating the *aroD* lifespan effect

Lifespan analysis was performed using the novel *aroD* mutant on both tryptone and peptone based media. Tryptone is a casein peptone used as it was not animal based, like peptone (Man 1960; Pham et al. 2005). Both types of media were used in order to determine whether the *aroD* mutant *E. coli* increased *C. elegans* lifespan more strongly on one type of media over the other. Data show that worms maintained on the *aroD* mutant living on average 25% longer than control on peptone media ($P<0.0001$) (Figure 9a), and 15% longer on tryptone media ($P=0.0006$) (Figure 9b). In the same experiment, the lifespan of worms maintained on *aroD E. coli* with 500 μM exogenous folic acid supplementation added to the plates was analysed. Data show that the lifespan was partially rescued with folic acid supplementation, but not

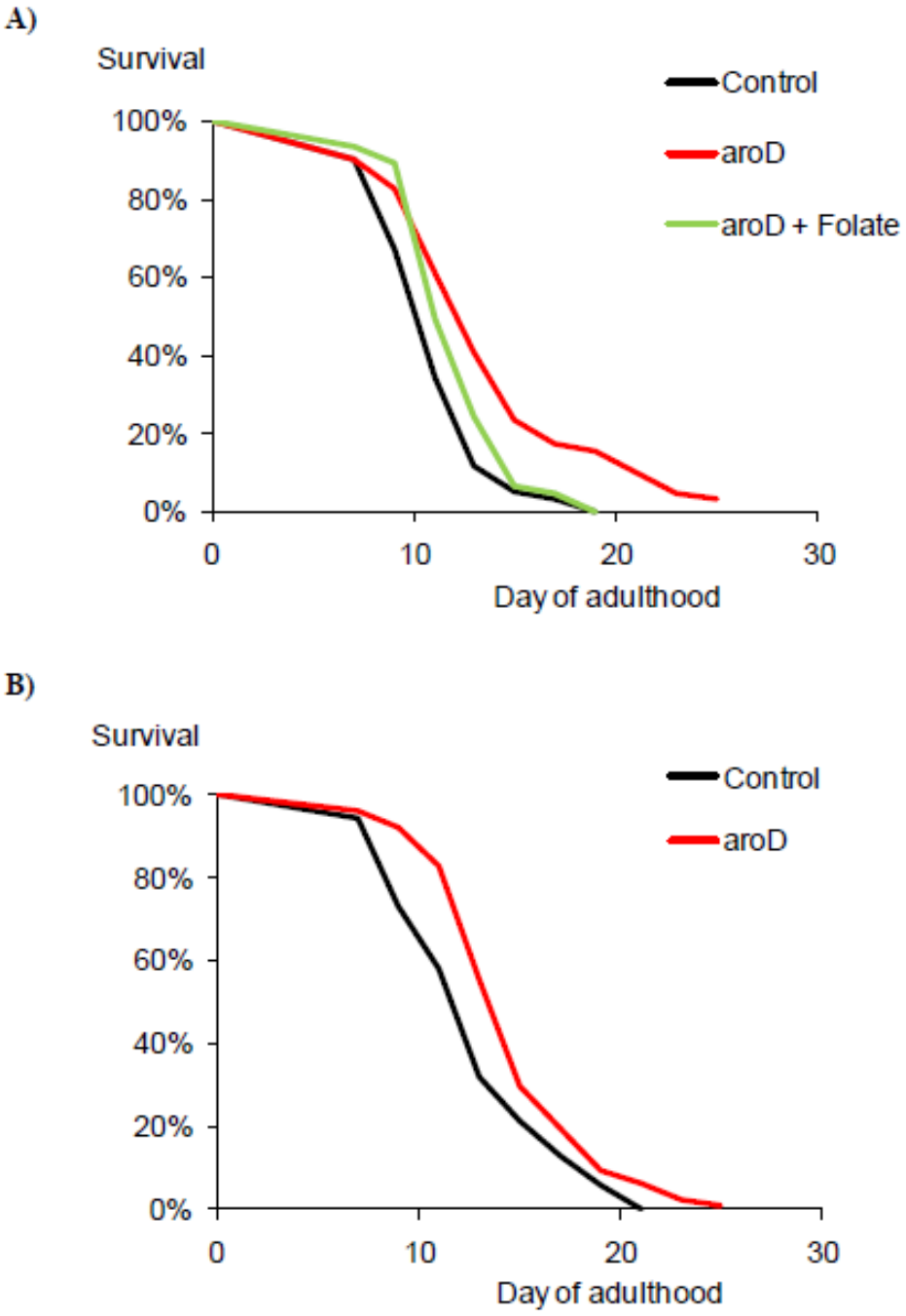


Figure 9: ***aroD E. coli*** **extends lifespan in** ***C. elegans.*** A) Lifespan analysis performed using rich agar and peptone media. *C. elegans* strain SS104, Control = HT115(DE3) wild type *E. coli*, *aroD* = HT115(DE3) *aroD* mutant *E. coli*. Folate concentration used = 500 µM. Control n = 119 mean lifespan = 11.24 days, *aroD* n = 72 mean lifespan 14.01 days (P<0.0001), *aroD* + Folate n = 45 mean lifespan 12.36 days (P=0.0246). B) Lifespan analysis performed using rich agar and tryptone media. *C. elegans* strain SS104, Control = HT115(DE3) wild type *E. coli*, *aroD* = HT115(DE3) *aroD* mutant *E. coli* Control n = 124 mean lifespan 12.94 days, *aroD* n = 97 mean lifespan = 14.85 days (P=0.0006). Data shown are representative of 2 biological replicates.

completely (Figure 9a), confirming previous findings. Whilst the *aroD* mutant did significantly increase lifespan in these lifespan analyses, the observed increase in lifespan was smaller than observed in previous studies in London and Durham, where the increase in lifespan caused by *aroD* was consistently between 30-50%. This suggests that either, the *aroD* mutant had lost the ability to increase animal lifespan – perhaps another mutation had occurred, or, the effect caused by the *aroD* mutant is sensitive to slight alterations in media, and there had been a change in the media.

3.2.2 Using growth assays to confirm the status of the *aroD* mutant

To validate the status of the *aroD* mutant by testing for auxotrophy for aromatic compounds, growth assays were performed on minimal media containing trace elements, aromatic amino acids and glucose. On this specific minimal medium (see section 2.4.3), the levels of folate present were not sufficient for the folate sensitive *aroD* mutant to grow. On this growth selective media, supplementation with 40 μg/ml (approximately equal to 250 μM) para-aminbenzoic acid (PABA) was able to rescue growth of the *aroD* mutant (Figure 10). This result is expected as *E. coli* are able to take up and utilise PABA in the media (Huang & Pittard 1967; Carter et al. 2007). Growth assays on minimal media showed that para-hydroxybenzoic acid (PHB) a precursor of quinones was able to rescue growth of the *aroD* mutant *E. coli* (Figure 11). This result was unexpected, as it suggested that the lifespan effect caused by *aroD* may be due to a lack of quinones similar to the GD1 *E. coli* (Saiki et al. 2008). Lifespan analysis showed that PHB did not rescue the *aroD* induced increase in *C. elegans* lifespan, and therefore the lifespan effect was not quinone specific (Figure 12)

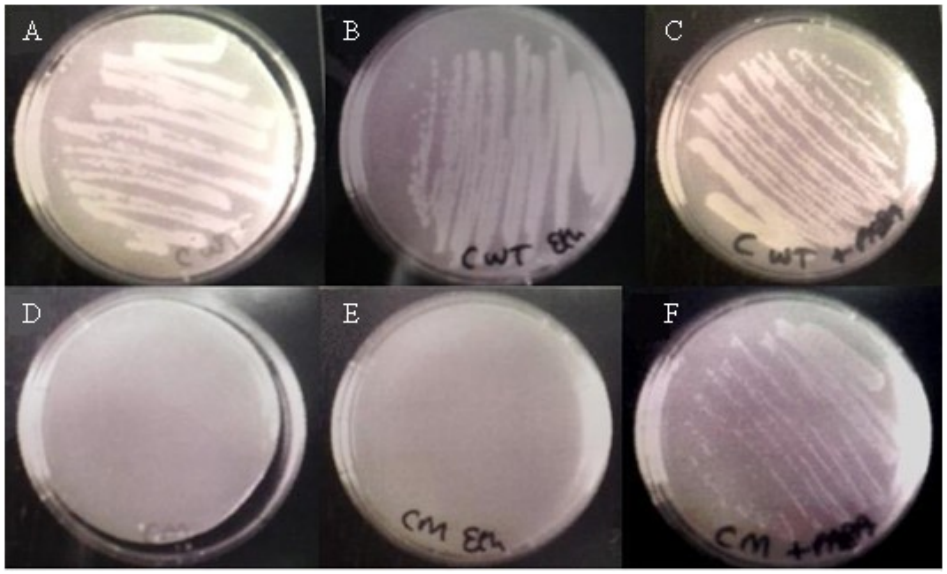


Figure 10: PABA rescues growth of *aroD* mutant on minimal media. PABA supplementation = 40µg/ml final concentration. Panels A-C show growth of wild type *E. coli* with no supplementation, ethanol supplementation and PABA supplementation respectively. Panels D-F show growth of the *aroD* mutant with no supplementation, ethanol supplementation and PABA supplementation respectively. All conditions were performed in triplicate with one representative plate shown.

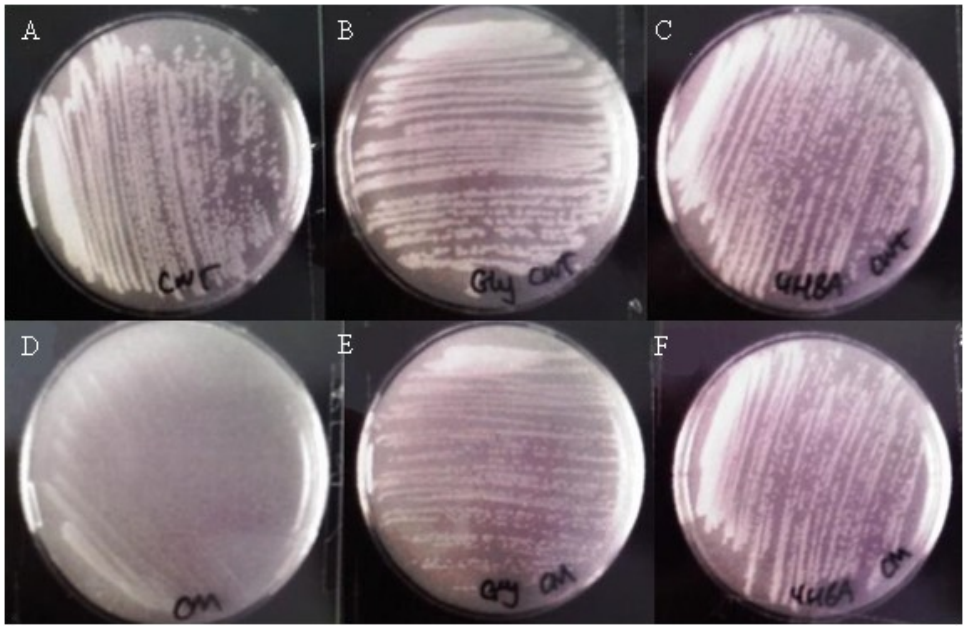


Figure 11: Both PHB and glycine rescue growth of *aroD* mutant on minimal media. PHB supplementation = 40µg/ml final concentration. Panels A-C show growth of wild type *E. coli* with no supplementation, glycine supplementation and PHB supplementation respectively. Panels D-F show growth of the *aroD* mutant with no supplementation, glycine supplementation and PHB supplementation respectively. All conditions were performed in triplicate with one representative plate shown.

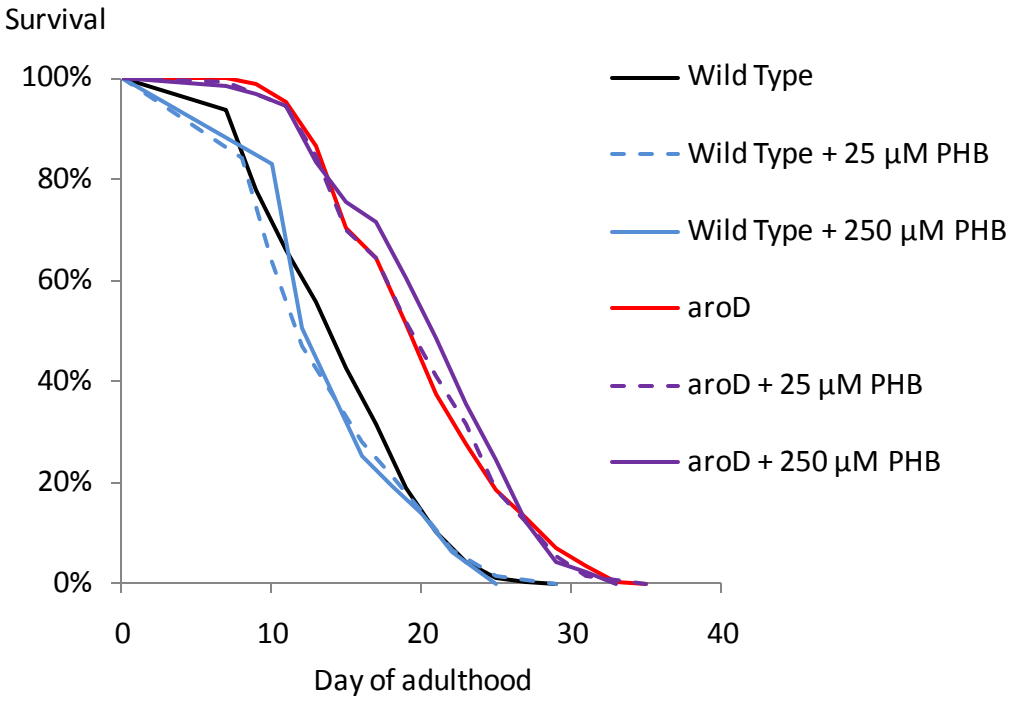


Figure 12: PHB does not rescue the increased lifespan observed in *C. elegans* maintained on *aroD E. coli*. Lifespan analysis performed on peptone high purity agar. *C. elegans* strain SS104, wild type = HT115(DE3) *E. coli, aroD* = HT115(DE3) *aroD* mutant *E. coli*. PHB was supplemented at the concentrations stated. Wild type n = 406 mean lifespan = 14.70 days, Wild type + 25 µM PHB n = 121 mean lifespan = 14.06 days (n.s), Wild type + 250 µM PHB n = 130 mean lifespan = 14.60 days (n.s), *aroD* n = 386 mean lifespan = 20.73 days (P<0.0001), *aroD* + 25 µM PHB n = 253 mean lifespan = 21.63 days (P<0.0001), *aroD* + 250 µM PHB n = 219 mean lifespan = 21.46 days (P<0.0001). Representative data is shown from 2 biological replicates.

Minimal media was used to determine whether folate supplementation would rescue the growth of the mutant *E. coli*, as it had been able to rescue the lifespan increase of worms maintained on the mutant *E. coli*. Minimal media was supplemented with the same high concentration of exogenous folic acid as used in lifespan analysis, 500 μM folic acid, and a lower concentration of 250 μM folic acid. Folate supplementation was able to rescue the growth of the *E. coli* (Figure 13), which was surprising as literature shows that *E. coli*, and other bacteria that make their own folate typically cannot take up exogenous folate (Shane & Stokstad 1975; Bedhomme et al. 2005).

3.2.3 Folic acid can breakdown into para-aminobenzoyl-glutamate (PAB-glu)

Further research into the literature shows that chemically synthesised folic acid can contain trace amounts of PAB-glu a breakdown product. PAB-glu can be taken up by bacteria and fed into the folate cycle (Carter et al. 2007). This could explain why folate was rescuing the growth of *E. coli*, and perhaps the partial rescue in lifespan effect seen with folic acid supplementation as well. As a result of this finding, we looked into alternate more specialised manufacturers of synthetics folates. We found a supplier, Schircks laboratories, which specialised in folate production.

3.2.4 Lifespan analysis with *aroD* on high purity agar

The growth assays show that the *aroD* mutant did not grow on the minimal media without para-aminobenzoic acid (PABA) supplementation. This suggests that the mutant has not changed or reverted, but I did not check the mutant by PCR or sequencing. Perhaps subtle changes in the media used for lifespan analysis was the reason that the mutant was causing a 15-25% increase in lifespan, rather than the expected 30-50% increase. The dilution of the *aroD* effect on lifespan correlated to a

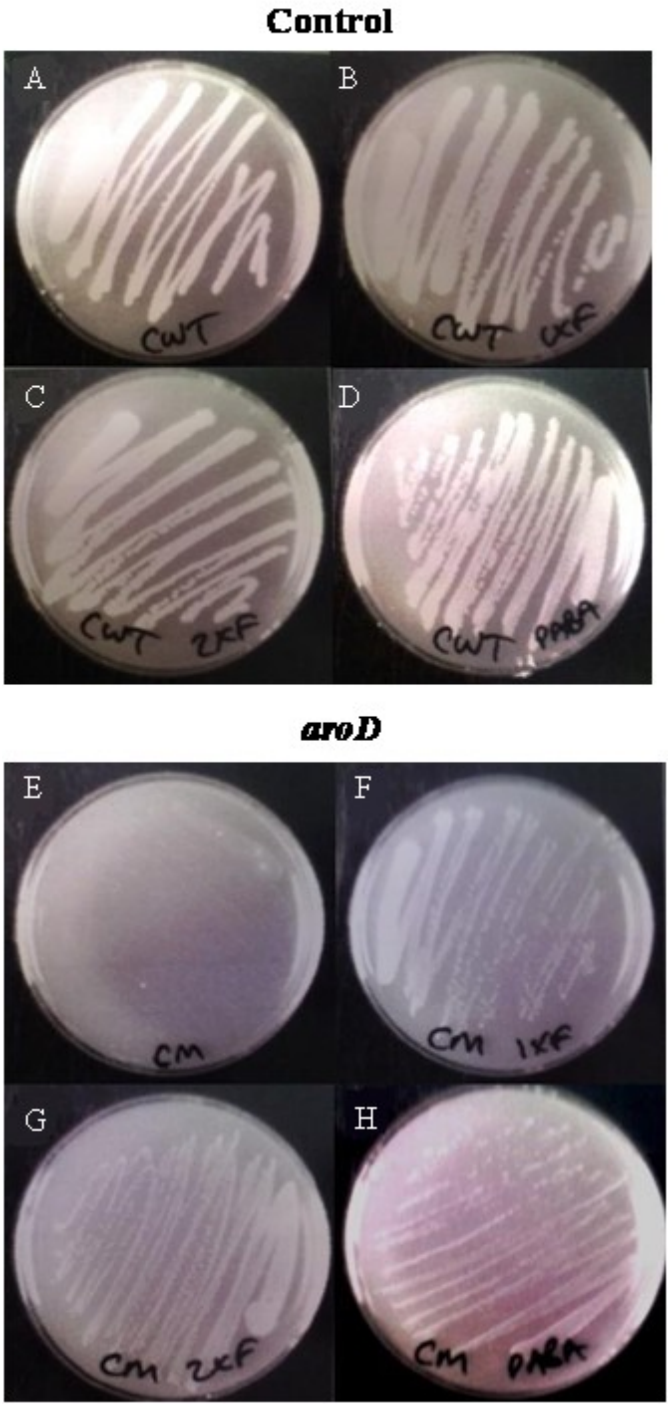


Figure 13: High concentrations of exogenous folate rescue growth of the *aroD* mutant on minimal media. Panels A-D show growth of wild type *E. coli* with no supplementation, 250 µM folic acid supplementation, 500 µM folic acid supplementation and PABA supplementation respectively. Panels E-H show growth of the *aroD* mutant with no supplementation, 250 µM folic acid supplementation, 500 µM folic acid supplementation and PABA supplementation respectively. PABA supplementation = 40 µg/ml final concentration. All conditions were performed in triplicate with one representative plate shown.

change in the batch of agar used in the lab. To address this question we ordered high purity agar, purified agar used in vitamin assays (05038, Sigma-Aldrich), and performed lifespan analysis with our novel mutant and an independent *aroD* mutant obtained from the Keio collection (Baba et al. 2006). The data show that on high purity media, our novel mutant significantly increased mean worm lifespan by 65% ($P<0.0001$), and the independent *aroD* mutant increased the mean lifespan of worms by 35%, also significant ($P<0.0001$) (Figure 14). These data confirm that the *aroD* mutant is media sensitive. Perhaps the medium used in previous lifespan analysis contained PAB-glu or a source PABA, which resulted in the *E. coli* using PABA to restore inhibited folate synthesis. If folate synthesis was restored, then it would be expected that an increase in lifespan would not be observed. Due to this finding, high purity agar was used for all further lifespan experimentation performed using the *aroD* mutant *E. coli*, in attempt to minimize sources of PABA available from the media.

3.2.5 The effect of PABA and folate supplementation on *aroD*

In light of the finding that synthetic folate can contain traces of PAB-glu which can then be used by *E. coli* to synthesise folates, we decided to perform lifespan analysis with lower amounts of folate supplementation, in attempt to reduce the availability of PAB-glu to the *E. coli*. Survival assays were performed with 250 μM, 100 μM, 25 μM and 10 μM folic acid, and also 100 μM and 10 μM folinic acid supplementation. Data show that 10 μM, 25 μM and 100 μM folic acid partially rescued the lifespan extension caused by the mutant *E. coli*, whereas the lifespan curve for 250 μM folic acid supplementation completely rescued the lifespan effect seen (Figure 15a). Both concentrations of folinic acid partially rescued the lifespan effect seen, showing a similar level of rescue to that of 10 μM and 25 μM folic acid (Figure 15b).

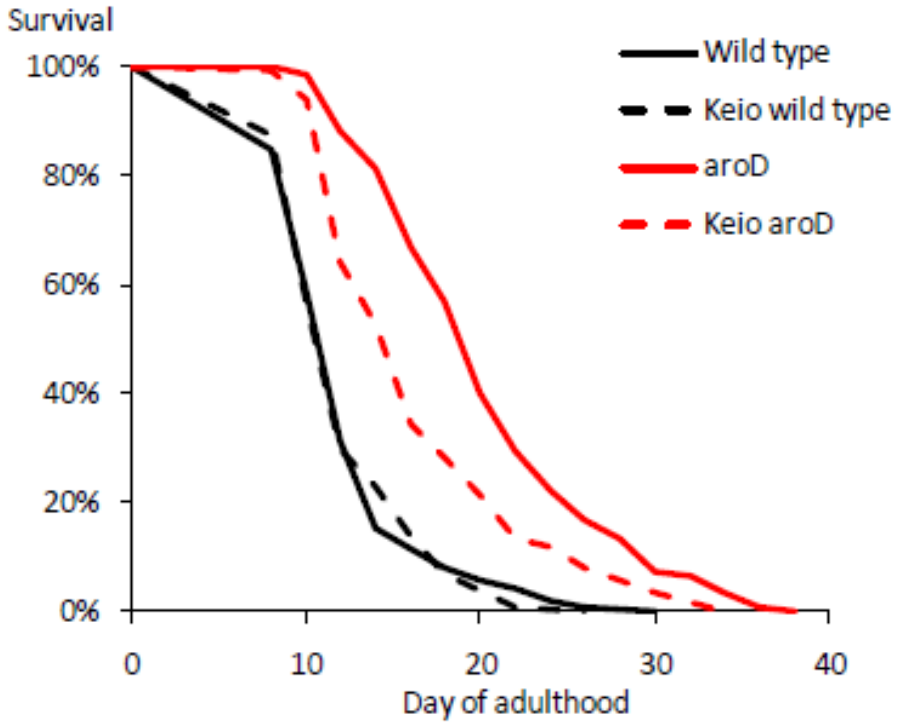


Figure 14: An independent *aroD* mutant from the Keio collection extends lifespan in *C. elegans*. Lifespan analysis performed using high purity agar and peptone media. *C. elegans* strain used SS104, Wild type = HT115(DE3) *E. coli, aroD* = HT115(DE3) *aroD* mutant *E. coli*. Keio represents equivalent strains from the Keio collection. Wild type n = 126 mean lifespan = 12.42 days, Keio wild type n = 131 mean lifespan = 12.45 days (n.s), *aroD* n = 127 mean lifespan 20.60 days (P<0.0001), Keio *aroD* n = 128 mean lifespan 16.76 days (P<0.0001). Representative data shown from 2 biological replicates.

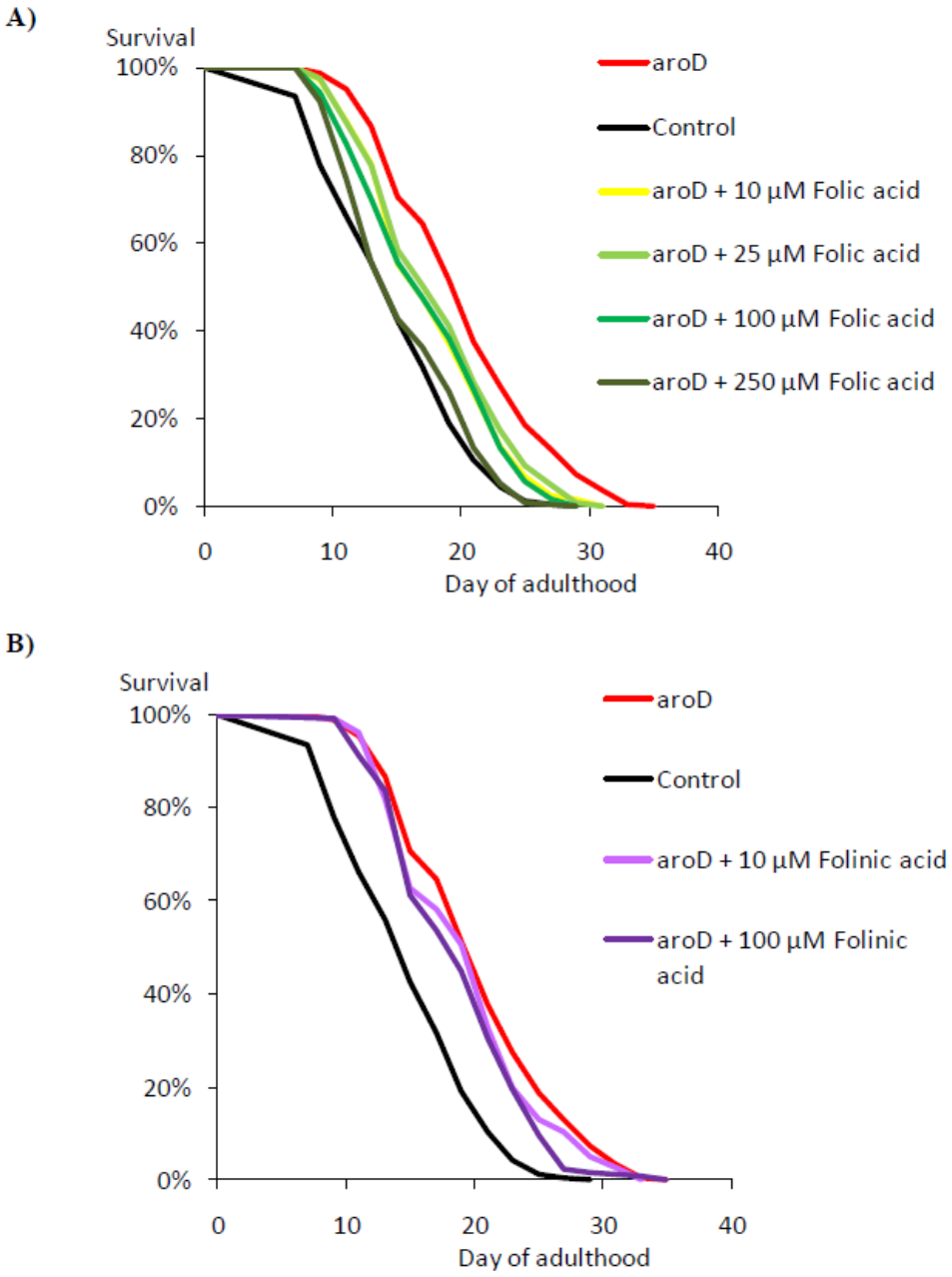


Figure 15: Exogenous folate partially rescues the increased lifespan of *C. elegans* maintained on *aroD* mutant *E. coli*. Lifespan analysis performed using high purity agar and peptone media with folic and folinic acid supplementation at the concentrations stated. *C. elegans* strain used SS104. Control = HT115(DE3) *E. coli, aroD* = HT115(DE3) *aroD* mutant *E. coli*. A) Folic acid supplementation: Control n = 233 mean lifespan = 15.06 days, *aroD* n = 225 mean lifespan = 20.51 days (P<0.0001), *aroD* + 10 µM folic acid n = 132 mean lifespan = 18.06 days (P<0.0001), *aroD* + 25 µM folic acid n = 127 mean lifespan = 18.46 days(P<0.0001), *aroD* + 100 µM folic acid n = 120 mean lifespan = 17.72 days (P<0.0001),, *aroD* + 250 µM folic acid n = 129 mean lifespan = 15.96 days (n.s). B) Folinic acid supplementation: Control and *aroD* same as used in A), *aroD* + 10 µM folinic acid n = 124 mean lifespan = 19.64 days (P<0.0001), *aroD* + 100 µM folinic acid n = 134 mean lifespan = 18.99 days (P<0.0001).

Lifespan analysis were also performed with PABA supplementation at 25μM and 250μM to determine whether lower levels of PABA would rescue the *aroD* lifespan effect. Data shows that both 25 μM and 250 μM completely rescue the increase in lifespan, without being toxic to the animals (Figure 16). Together these data show that the lifespan increase observed when *C. elegans* are maintained on *aroD* mutant *E. coli* is folate specific.

3.2.6 A drug can be used to target folate synthesis and increase *C. elegans* lifespan

The *aroD* gene is involved in the production of chorismate in the early stages of the shikimic acid pathway. As data suggest that the lifespan effect caused by *aroD* mutant *E. coli* is specific to folate synthesis, it raised the question, whether this folate specific lifespan increase was unique to *aroD E. coli* strain, or could it be applied to other bacterial strains. I also aimed to find an intervention to folate synthesis that showed a more robust response with regards to the media used. I used sulfamethoxazole (SMX) as a chemical intervention to inhibit folate synthesis in the *E. coli* strain OP50, the strain of bacteria typically fed to *C. elegans* in the lab. Following the preliminary research performed by Andrea Bender, for the purpose of this research three concentrations of SMX were chosen for use. These were 16 μg/ml, 64 μg/ml and 128 μg/ml SMX as these represented a low, medium and high dose based on the previous study. Survival assays using these 3 concentrations of SMX to treat the OP50 *E. coli* were performed on the same standard, not high purity agar that had been used for the initial *aroD* lifespans. On tryptone based media, results show that all concentrations of SMX used increase *C. elegans* lifespan significantly. Supplementation with 128 μg/ml SMX showed the largest increase in lifespan of 26% ($P<0.0001$), with 64 μg/ml SMX

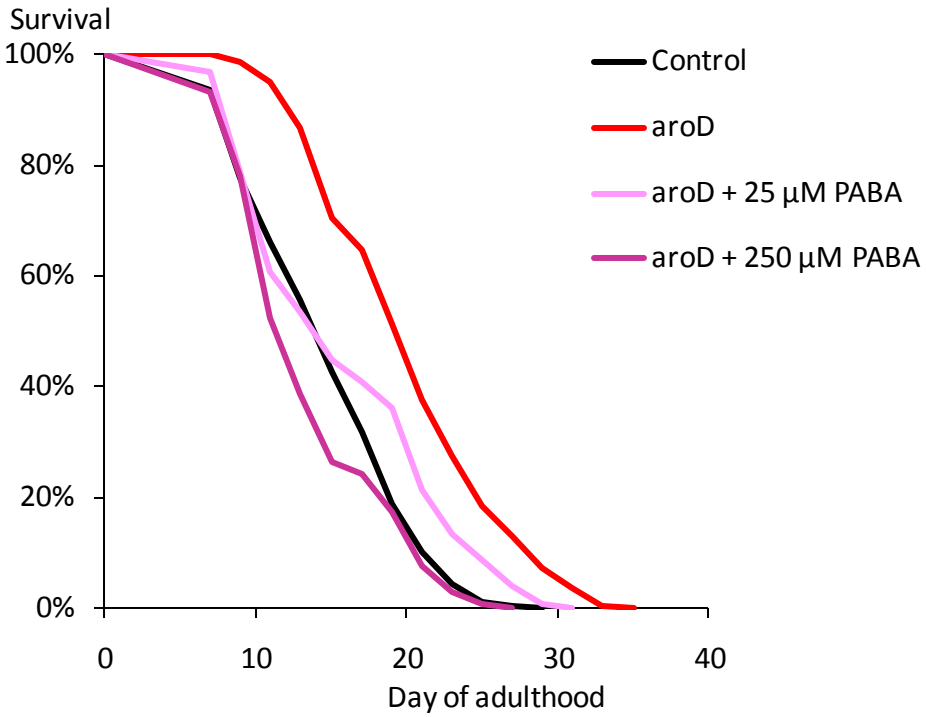


Figure 16: A high concentration of PABA supplementation completely rescues the increased lifespan of *C. elegans* maintained on *aroD* mutant *E. coli*. Lifespan analysis performed using high purity agar and peptone media with PABA supplementation at the concentrations stated. *C. elegans* strain used SS104. Control = HT115(DE3) *E. coli*, *aroD* = HT115(DE3) *aroD* mutant *E. coli*. Control n = 233 mean lifespan = 15.06 days, *aroD* n = 225 mean lifespan = 20.51 days (P<0.0001), *aroD* + 25 µM PABA n = 127 mean lifespan = 16.20 days (n.s), *aroD* + 250 µM PABA n = 132 mean lifespan = 13.83days (n.s). Data shown is representative from 2 biological replicates.

supplementation causing a 13% increase (Log Rank P=0.0107, Wilcoxon P=0.0028), and 16 μg/ml SMX supplementation with a similar increase of 16% (Log Rank P=0.0002, Wilcoxon P=0.0003) (Figure 17a). On peptone media, only 128 μg/ml SMX was supplemented and compared to the control group. 128 μg/ml SMX supplementation showed a 46% increase in longevity (P<0.0001) (Figure 17b).

3.2.7 Lifespan analysis with SMX on high purity agar

Lifespan analysis was also performed with these 3 concentrations of SMX supplementation on the high purity agar obtained. On the tryptone media, again 128 μg/ml supplementation showed the largest increase at 28% (P<0.0001), with 64 μg/ml SMX supplementation causing a 25% increase (Log Rank P=0.0029, Wilcoxon P<0.0001), and 16 μg/ml SMX supplementation with an increase of 19% (Log Rank P=0.0317, Wilcoxon P=0.0003) (Figure 18a). On the peptone based high purity media only 128 μg/ml was supplemented and compared to the control. 128 μg/ml supplementation showed a 63% increase in longevity (P<0.0001) (Figure 18b). On the higher purity agar the longevity increases observed are larger than on the standard agar. The lifespan increase induced by SMX treatment supports the preliminary data obtained by Andrea, and confirms that SMX treatment increase *C. elegans* lifespan.

3.2.8 The effect of PABA and folate supplementation on SMX

In order to investigate whether the longevity increase caused by SMX was folate specific, lifespan experiments were performed with 16 μg/ml and 128 μg/ml SMX supplementation, and supplementation with PABA. As shown in Figure 19, supplementation with PABA at a concentration of 250 μM rescued the lifespan increase caused by SMX completely. The data also indicated that PABA

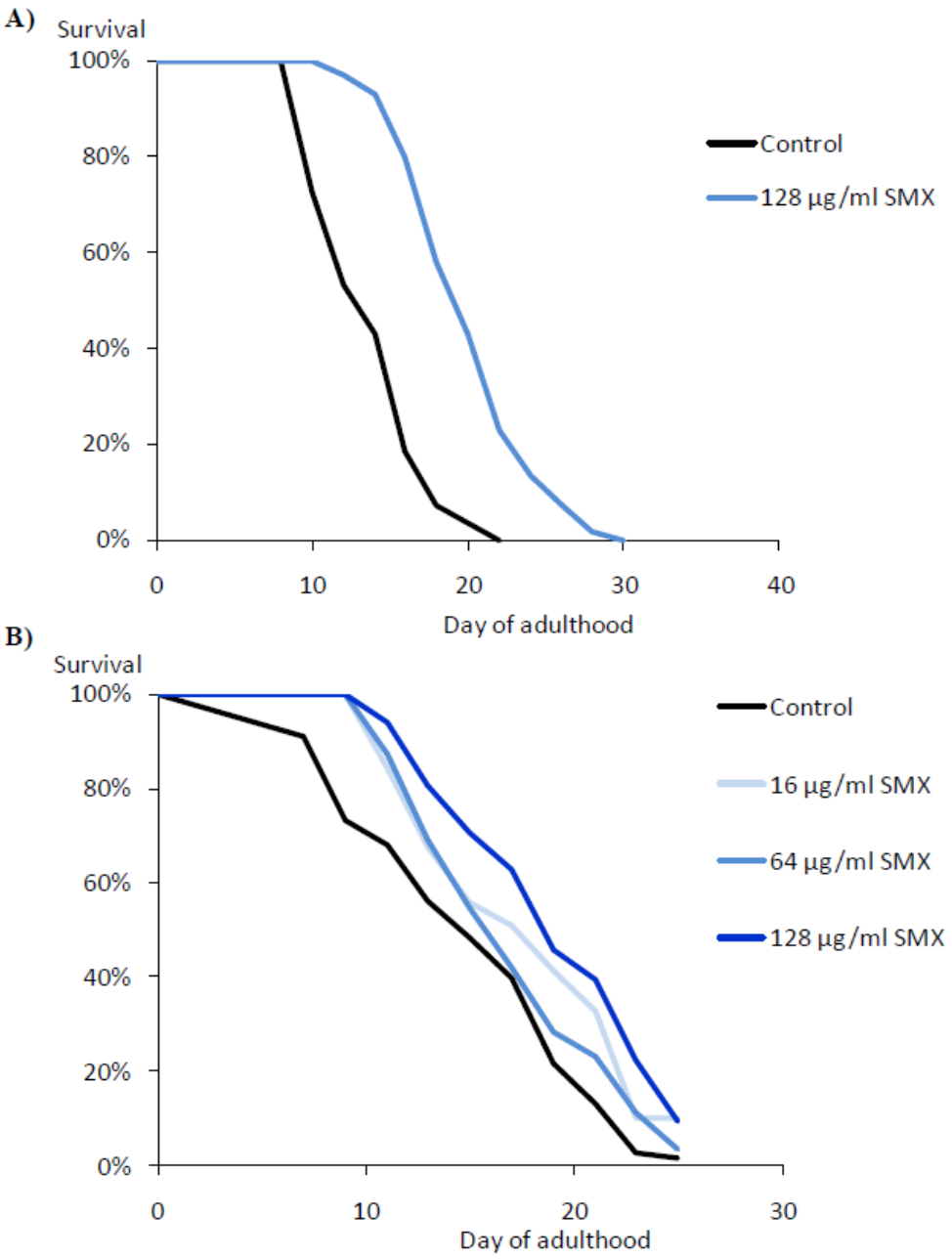


Figure 17: SMX-treated *E. coli* extends *C. elegans* lifespan. *C. elegans* strain used SS104. Control = OP50 *E. coli*. A) Lifespan analysis performed using peptone media and rich agar. Control n = 116 mean lifespan = 13.95 days, 128 µg/ml SMX n = 128 mean lifespan = 20.30 days (P<0.0001). B) Lifespan analysis performed using tryptone media and rich agar, with SMX supplementation at the concentrations stated. Control n = 115 mean lifespan = 15.27 days, 16 µg/ml SMX n = 78 mean lifespan = 17.61 days (P=0.0003), 64 µg/ml SMX n = 113 mean lifespan = 17.31 days (P=0.0107), 128 µg/ml SMX n = 102 mean lifespan 19.32 days (P<0.0001).

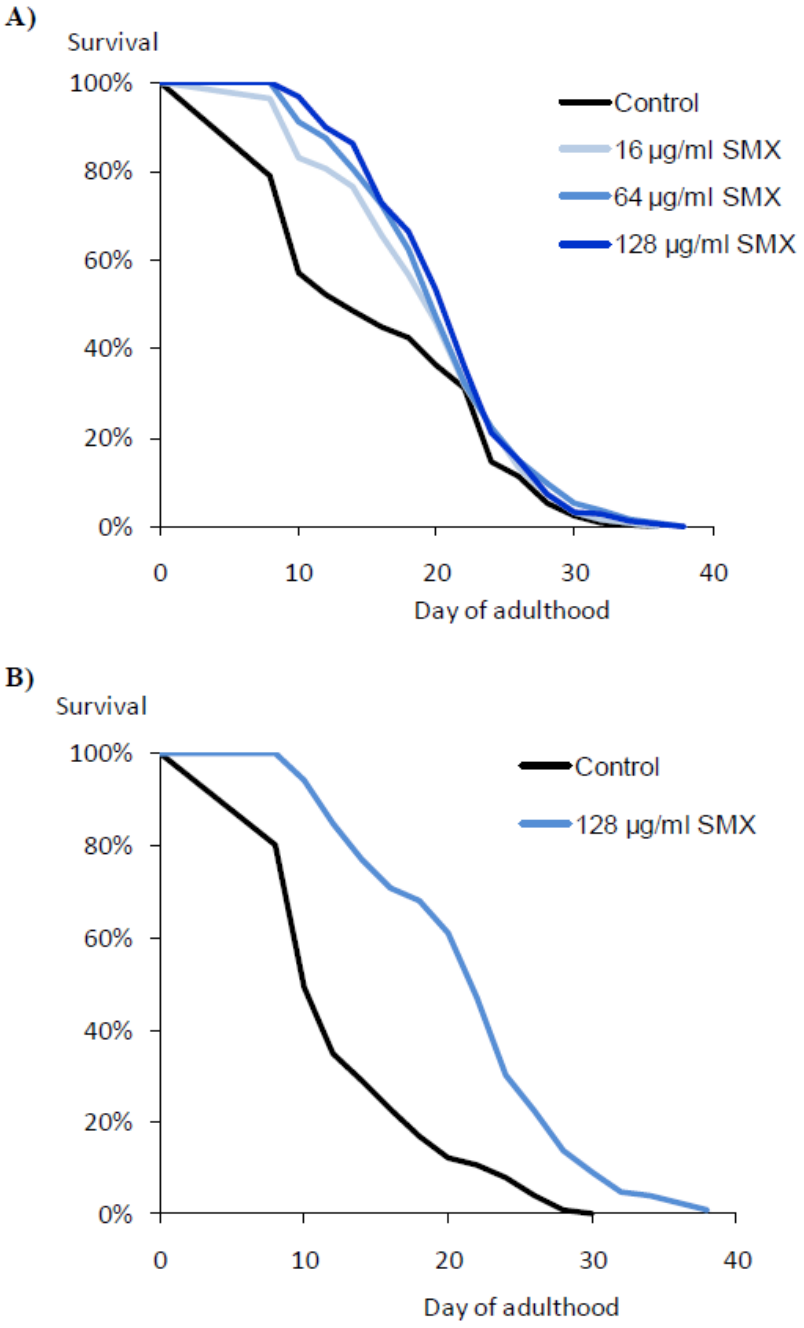


Figure 18 SMX-treated OP50 extends animal lifespan on both tryptone and peptone media. *C. elegans* strain used SS104. Control = OP50 *E. coli*. A) Lifespan analysis performed using tryptone media and high purity agar, with SMX supplementation at the concentrations stated. Control n = 132 mean lifespan = 16.53 days. 16 µg/ml SMX n = 122 mean lifespan = 19.72 days (P=0.0317), 64 µg/ml SMX n = 135 mean lifespan = 20.64 days (P=0.0029), 128 µg/ml SMX n = 152 mean lifespan 21.08 days (P<0.0001). B) Lifespan analysis performed using peptone media and high purity agar. Control n = 127 mean lifespan = 13.36 days, 128 µg/ml SMX n = 129 mean lifespan 21.81 days (P<0.0001).

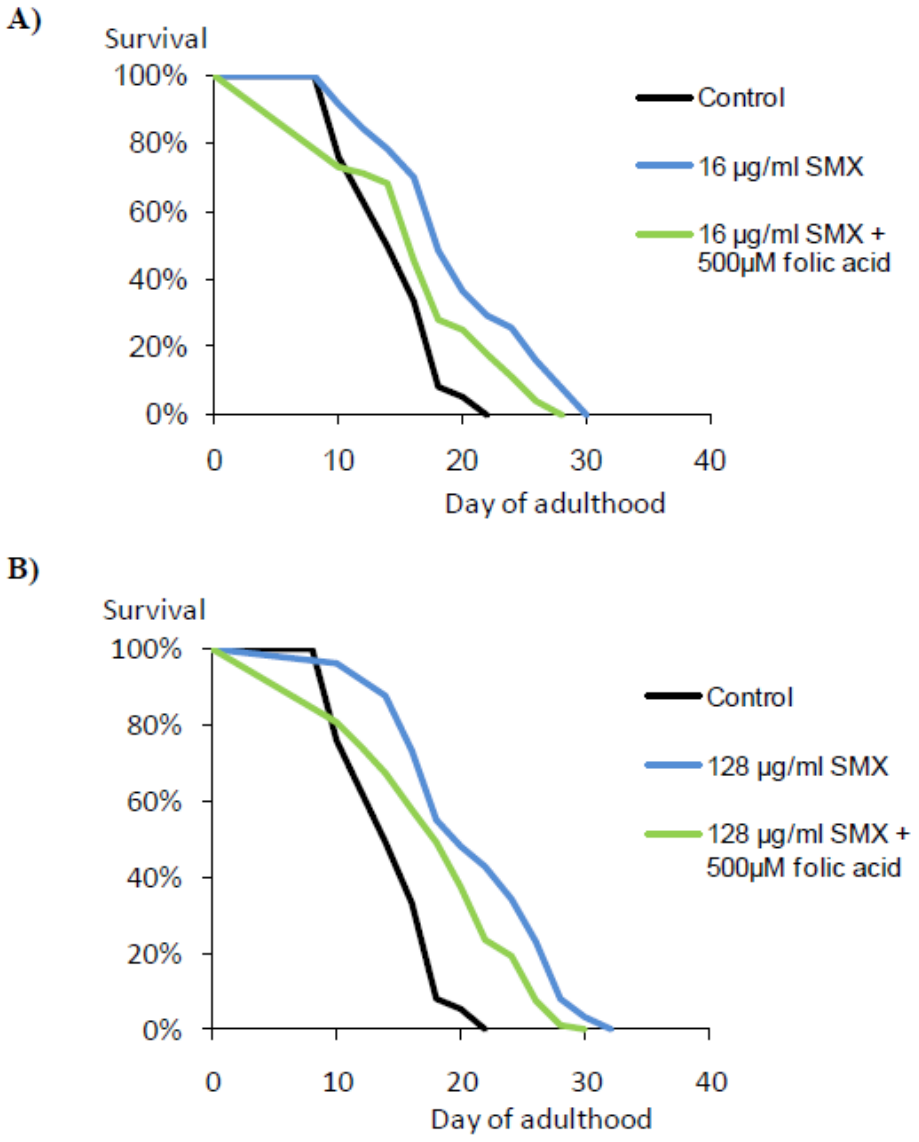


Figure 19: Exogenous folic acid partially rescues the lifespan extension in *C. elegans* maintained on SMX-treated OP50 *E. coli*. Lifespan analysis performed using tryptone media and rich agar, with SMX supplementation and folic acid at the concentrations stated. *C. elegans* strain used SS104. Control = OP50 *E. coli*. Control for A) and B) n = 75 mean lifespan = 14.69 days. A) 16 µg/ml SMX n = 118 mean lifespan = 19.74 days (P<0.0001), 16 µg/ml SMX + 500 µM folic acid n = 97 mean lifespan = 16.86 days (P=0.0002) B) 128 µg/ml SMX n = 122 mean lifespan 21.27 days (P<0.0001), 128 µg/ml SMX + 500 µM folic acid n = 107 mean lifespan = 18.35 days (P<0.0001).

supplementation was not toxic or detrimental to the worm as in the *aroD* PABA rescue lifespan experiment. These data support the hypothesis that the lifespan effect caused by SMX is indeed folate specific.

To further investigate whether the SMX was increasing lifespan via a folate specific pathway, the low and high concentrations of SMX, 16 μg/ml and 128 μg/ml were used for lifespan analysis with high exogenous folic acid supplementation (500 μM), to investigate whether the folic acid would rescue lifespan. Lifespans were performed on standard (not high purity) agar and tryptone media. Folate supplementation resulted in partial rescue of both concentrations of SMX used (Figure 20), similar to the *aroD* mutant *E. coli*, suggesting that SMX is extending lifespan via a folate specific mechanism.

Once it had been confirmed that SMX did indeed increase lifespan in a robust fashion independent of media, it was decided that for consistency all further experiments would be performed on high purity agar, in order to reduce batch to batch variation, with peptone based media. To determine whether there was a threshold level at which the lifespan extension can be observed, or at which it plateaus, a range of SMX concentrations from 0.1 μg/ml – 256 μg/ml were used to supplement media in lifespan assays. The data shows that SMX supplementation causes the worms live longer, with 2 μg/ml SMX causing a significant increase in lifespan compared to the control (Replicate 1: 14% increase Log rank $P<0.0001$, Wilcoxon $P=0.0005$, Replicate 2: 15% increase $P<0.0001$). Concentrations of SMX below 2 μg/ml did increase lifespan, but not substantially enough to be statistically significant. A dose of 128 μg/ml SMX produced the largest and most robust increase (Replicate 1: 53% increase $P<0.0001$,

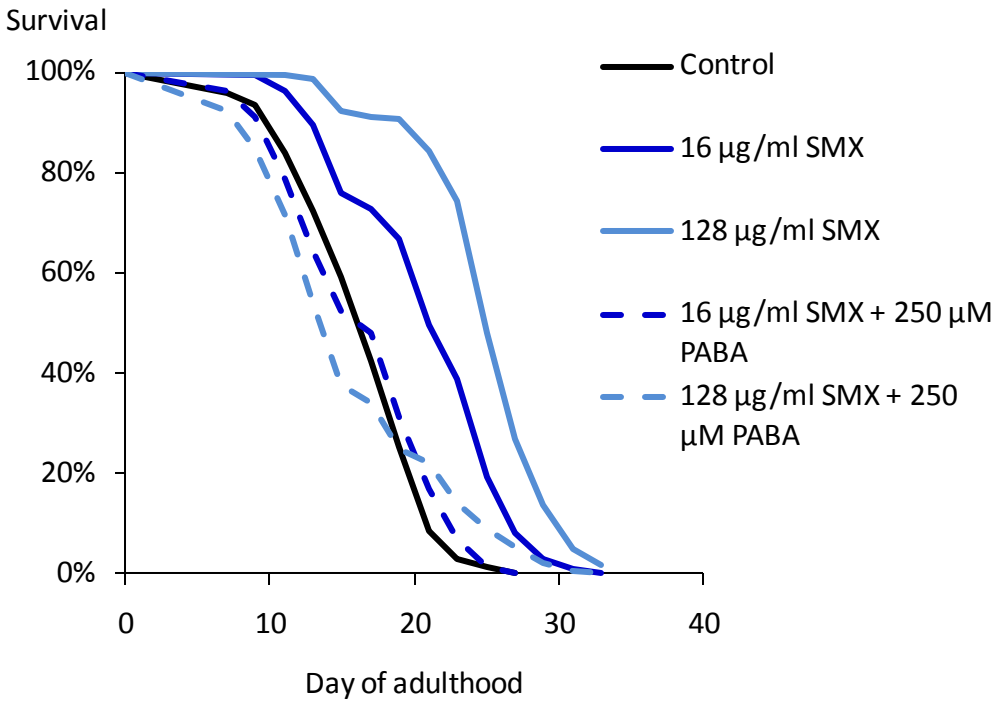


Figure 20: A high concentration of PABA supplementation completely rescues the increased lifespan of *C. elegans* maintained on SMX-treated *E. coli* Lifespan analysis performed using peptone media and high purity agar, with SMX and PABA supplementation at the concentrations stated. *C. elegans* strain used SS104. Control = OP50 *E. coli*. Control n = 102 mean lifespan = 16.70 days. 16 µg/ml SMX n = 176 mean lifespan = 21.41 days (P<0.0001), 16 µg/ml SMX + 250µM PABA n = 160 mean lifespan = 16.75 (n.s), 128 µg/ml SMX n = 229 mean lifespan 25.49 days (P<0.0001), 128 µg/ml SMX + 250µM PABA n = 217 mean lifespan = 16.05 days (n.s). Representative data shown from 2 biological replicates.

Replicate 2: 52% increase P<0.0001) (Figure 21).

3.2.9 Both the *aroD* mutant and SMX treatment extend animal longevity in a folate specific manner

Together with the *aroD* lifespan data, and in light of the knowledge that man made folic acid can contain the breakdown product para-aminobenzoyl-glutamate (PAB-glu), the data obtained strongly suggest that the extension in lifespan seen was due to mutation of *aroD* in *E. coli* and the SMX treatment of the *E. coli*. If this were the case, it would lead to the idea that the folate synthesis and folate levels in *E. coli* are important with regards to worm lifespan. In fact it could be hypothesised that neither the *aroD* mutation nor SMX supplementation appear to have any effect in the worm itself, but rather in the *E. coli*, and that the lifespan effect is a result of the worms eating this physiologically different *E. coli*.

3.2.10 *C. elegans* maintained on SMX resistant OP50 show no lifespan extension

To test this hypothesis, that the SMX was having an effect mainly on the *E. coli* further lifespan analysis was performed using SMX-resistant OP50. In this study a strain of OP50 that had been transformed using a protocol from Prof. John Ward (UCL), so that it possessed the R26 plasmid. This OP50 R26 *E. coli* strain was used for lifespan analysis, rather than the standard strain of OP50 usually used. The R26 plasmid confers resistance to several antibiotics, including sulfonamides as it has a drug insensitive allele of dihydropteroate synthase (Stanisich et al. 1976). Data show that when *C. elegans* were maintained on sulfonamide resistant *E. coli* regardless of whether the plates have been supplemented with SMX, there was no lifespan extension seen (Figure 22). This result supports the hypothesis that SMX treats, and

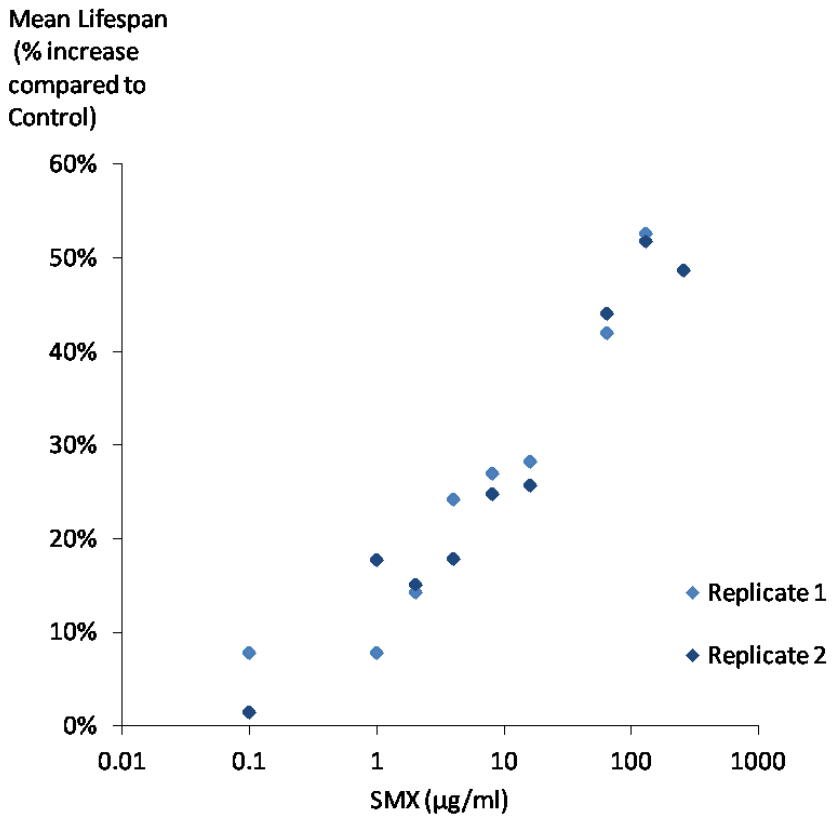


Figure 21: SMX supplementation results in a dose dependent increase in *C. elegans* lifespan. Lifespans performed on high purity agar and peptone media. *C. elegans* strain used SS104. *E. coli* strain = OP50 *E. coli*. Percentage increase in mean lifespan as compared to control for 2 replicates are shown. Control n = 102, 191, 0.1 µg/ml SMX n = 139, 191, 1 µg/ml SMX n = 154, 229, 2 µg/ml SMX n = 152, 210, 4 µg/ml SMX n = 161, 226, 8 µg/ml SMX n = 146, 224, 16 µg/ml SMX n = 176, 226, 64 µg/ml SMX n = 235 238, 128 µg/ml SMX n = 229, 230, 256 µg/ml SMX n = 253.

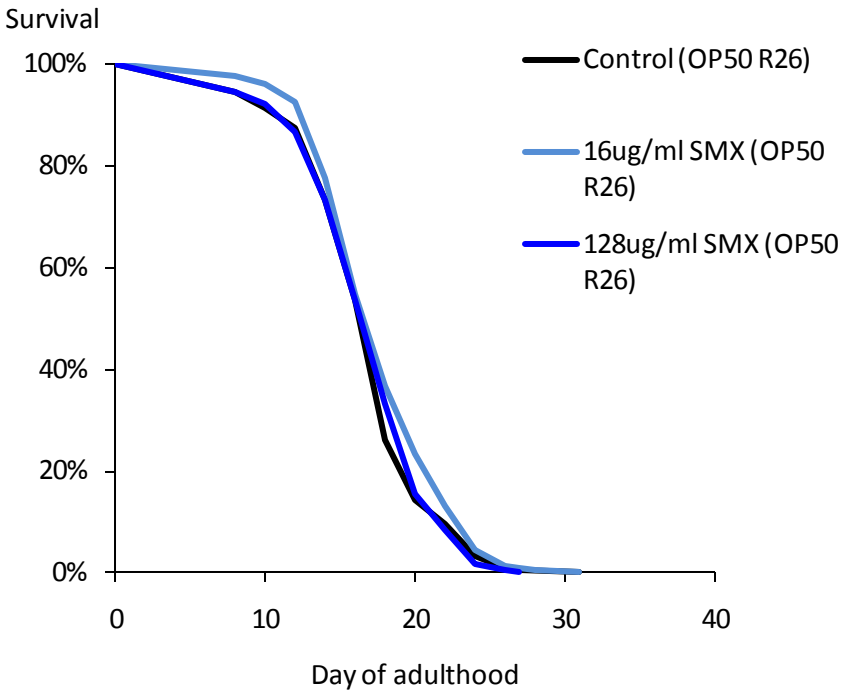


Figure 22: SMX treatment does not extend animal longevity in sulfonamide resistant *E. coli*. Lifespan performed on high purity agar and peptone media. *C. elegans* strain used SS104, *E. coli* strain OP50 R26 was used, as the R26 plasmid confers resistance to sulfonamides. Control n = 215 mean lifespan = 17.11 days, 16 µg/ml SMX n = 218 mean lifespan = 17.98 days (n.s), 128 µg/ml SMX n = 224 mean lifespan = 17.19 days (n.s).

has its effect on the *E. coli*, and it is these treated *E. coli* that cause a lifespan extension in the animal.

3.3 Discussion

In this chapter I have shown that the *aroD* mutant *E. coli* extends worm longevity. However, the lifespan effect is variable and sensitive to the type of media being used. A more consistent lifespan effect, would allows me to delve further into the mechanism behind which this mutant *E. coli* is increasing lifespan. The targeted pharmalogical intervention in the form of SMX that I have applied does extend *C. elegans* lifespan in a more robust, consistent and dose dependent manner, regardless of the type of media or agar. I have also shown that the lifespan effect in both *aroD* mutant *E. coli* and with SMX supplementation appears to be folate specific, and not to do with effects on the worm. The addition of PABA supplementation completely rescues the increased longevity induced by both *aroD* and SMX alike, and supplementation with exogenous folic acid partially rescues the extension in lifespan.

Folates are very important for cell growth and repair as well as purine synthesis required for DNA and RNA synthesis. In both the *aroD* mutant and the SMX supplementation I am inhibiting the folate cycle of the *E. coli* but they are still able to form a lawn on the plates used for lifespan. Formation of the lawn suggests that even with inhibition of folate synthesis, there are sufficient levels of folate for growth. Observation of the presence of a lawn by eye is a crude measurement for bacterial growth however, and will not detect subtle growth defects.

Furthermore *C. elegans* are unable to synthesise folates themselves and rely on microbes for folate uptake. If folate synthesis in the *E. coli* is inhibited, then the worm *C. elegans* will be taking up less folate. It is possible therefore, that the lifespan effect seen in the two systems above is a form of dietary restriction. Whilst performing lifespan assays with SMX supplementation, there was a clear difference between plates that had SMX supplemented and the control. On the non-supplemented plates the worms were evenly distributed over the area of the plate regardless of where the lawn was. On the contrary on plates that contained a moderate SMX supplementation (a concentration of 2 μg/ml or above), the vast majority of the animals were either in the lawn or on the edge of the lawn. The worms appeared to prefer the SMX-treated OP50 to normal OP50, which suggests that perhaps it isn't a DR effect, at least not in the classic sense.

CHAPTER 4

EFFECTS OF SULFAMETHOXAZOLE ON *E. COLI* AND *C. ELEGANS*

4.1 Introduction

C. elegans maintained on sulfamethoxazole (SMX)-treated OP50 *E. coli* show a robust increase in lifespan. As described in chapter 3, data suggest that the lifespan increase is folate specific, as the extension in longevity can be fully rescued with para-aminobenzoic acid (PABA) supplementation. The mechanism by which SMX causes increased *C. elegans* longevity, whilst folate dependent, remains unclear. SMX is a bacteriocidal antibiotic (Kobayashi et al. 1978; Weening et al. 1983; Yeldandi et al. 1988) and therefore it may be that SMX prevents *E. coli* proliferation. If this were so then SMX may be working via a dietary restriction mechanism, less food for worms results in less folate, and worms depends on *E. coli* for folates. The inhibition of folate synthesis by SMX treatment in the *E. coli* may also cause growth defects, due to the lack of folate species which are required for healthy growth and cell development (Bailey & Gregory 1999; Hjortmo et al. 2008; Blatch et al. 2010)

4.1.1 Folate quantification

The analysis and quantification of folates can be performed using many methods. Such methods include microbiological assays, in which folates are quantified by measuring the growth of *Lactobacillus rhamnous*, liquid chromatography (LC), and mass spectrometry (Garratt et al. 2005; Lu et al. 2007; Scott et al. 2000; Kwon et al. 2008a). Coupling LC with mass spectrometry (LC-MS) allows the distinction between different folate forms such as dihydrofolate (DHF) or tetrahydrofolate (THF) as

described in chapter 1 (Figure 2), resulting in LC-MS becoming a leading tool with regards to analysis of folate species (Lu et al. 2007). Further development using LC-MS, resulted in the ability to detect large numbers of both mono- and polyglutamated folates and their molecular precursors present in small amounts of plant material. Following these findings, Lu et al. were able to profile the pool of folate species present in *E. coli* using LC-MS(Garratt et al. 2005; Lu et al. 2007; Kwon et al. 2008a).

If sulfamethoxazole (SMX) increases lifespan via dietary restriction (DR), then it is possible that there is a trade off in the worm with regards to the longevity increase induced when *C. elegans* are fed *E. coli* treated with SMX. Thus far, only the lifespan of adult worms maintained on SMX-treated *E. coli* has been observed and not any traits earlier in life during development. It may be that an extension in lifespan comes at a cost of slow development, as observed with DR. As folates are required for many essential processes during development, such as purine synthesis and methionine regeneration (Bailey & Gregory 1999; Hjortmo et al. 2008; Blatch et al. 2010), it may be that SMX causes deleterious effects on the development and/or reproductive cycle of the worm alongside the increase in lifespan. If such a trade off were occurring, then whilst *C. elegans* may live long with SMX supplementation, the health span and thus quality of life may be suffering.

4.1.2 Aims and Objectives

In this chapter I aim to investigate changes in metabolism, in both *E. coli* and *C. elegans* caused by SMX. In order to provide further information into any metabolic or physiological changes caused by SMX supplementation, folate levels will be measured and quantified using LC-MS, in both worms and *E. coli*. The folate status of

OP50 *E. coli* and SMX-treated *E. coli* will be determined, as will the folate levels in both *C. elegans* fed on OP50 *E. coli*, and those fed on SMX treated OP50. I will also use mass spectrometry to investigate changes in folate levels induced by the *aroD* mutant *E. coli*. I intend to investigate whether the SMX induced lifespan increase in *C. elegans* causes any deleterious effects on growth or fecundity.

4.2 Results

4.2.1 Folate analysis of *aroD E. coli*

In order to be confident that the increase in *C. elegans* lifespan observed with SMX supplementation and the *aroD* mutant *E. coli* is caused by a decrease in folate levels specifically, together with an international exchange student Nikolin Oberleitner, I adopted folate extraction methods from published literature (Garratt et al. 2005; Lu et al. 2007) in attempt to quantify folate levels in *E. coli*. The *E. coli* folate extraction protocol used initially was unable to detect folate species, and therefore Nikolin and I adapted the extraction protocol. The new extraction procedure, as described in chapter 2 (section 2.13.2), was performed on wild type HT115 (DE3) *E. coli* and the *aroD* mutant *E. coli* grown on plates at 25°C. The most abundant folate in these bacterial samples was formyl-tetrahydrofolate with 3 glutamates (formyl- $THFGlu_3$), in previous literature, methyl-tetrahydrofolate with 3 glutamates (methyl- $THFGlu_3$) was shown to be the most abundant folate detected (Lu et al. 2007). The resulting chromatograms were integrated, and the area under the peak used as a measure of how much of a particular folate species was present. To allow comparison between different samples, a ratio of amount of folate compared to the amount of Coenzyme A (CoA) present was used. It is assumed that CoA is at similar levels across the different

samples. My data show that levels of formyl- $THFGlu_3$ are significantly lower in *aroD E. coli* as compared to the wild type HT115(DE3) *E. coli* (Figure 23).

4.2.2 Folate analysis of *C. elegans* maintained on *aroD E. coli*

The *E. coli* folate extraction protocol was carried out on *C. elegans*, however using this method, it was not possible to detect many folate species present in the worms. A search of the relevant literature revealed that folate extraction in *C. elegans* had not been performed previously. The method for worm folate extraction was optimised by a summer student, Gonçalo Correia, and differs from *E. coli* folate extraction in that proteinase K and glass beads are used to aid digestion of the *C. elegans* (section 2.13.3). The optimised worm folate extraction protocol was carried out on *C. elegans* maintained on wild type *E. coli*, and those maintained on the *aroD* mutant. In these worm extraction samples, folate species were detected, the most abundant of which was 5-methyl-tetrahydrofolate with 5 glutamates (5m-$THFGlu_5$). To the best of my knowledge, folate levels had not been measured in *C. elegans* previously. Results show that levels of 5m-$THFGlu_5$ are significantly lower in *C. elegans* maintained on *aroD* mutant *E. coli*, than in *C. elegans* maintained on wild type *E. coli* (Figure 24).

These findings confirm that folate levels are decreased in *aroD E. coli* and in worms maintained on *aroD E. coli*, and therefore it could be that a decrease in folate levels is the cause of the lifespan extension observed in *C. elegans* maintained on *aroD E. coli*. In *aroD E. coli*, the decrease in folate levels, compared to wild type *E. coli* is much more severe than the decrease in folate levels of worms maintained on *aroD E. coli*, and worms maintained on wild type *E. coli*. This suggests that even though *aroD*

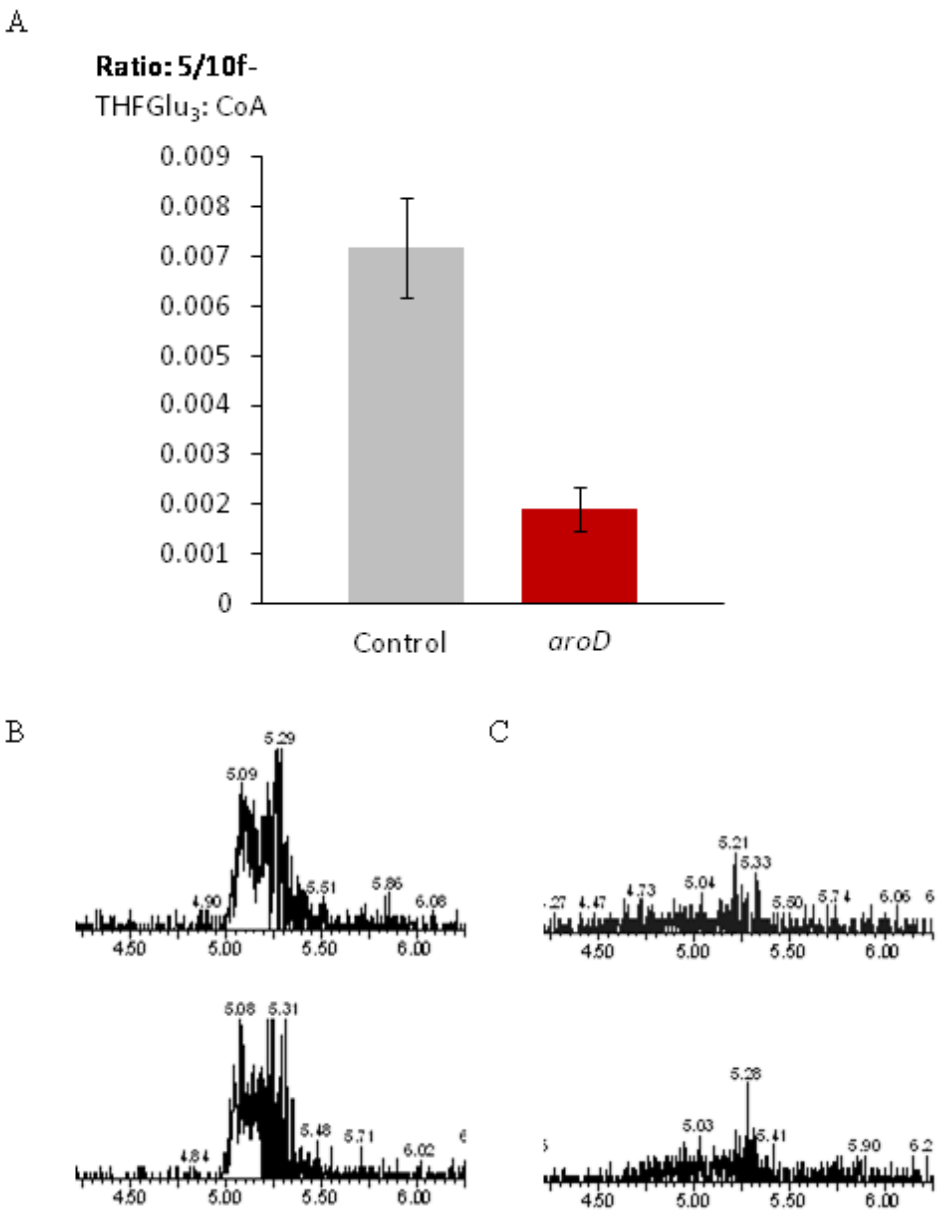


Figure 23: Levels of formyl-THFGlu$_3$ detected by LC-MS are decreased in the *aroD* mutant *E. coli* compared to the wild type HT115(DE3). Data shown are from two biological replicates on high purity agar and peptone media. B) and C) show the formyl-THFGlu$_3$ peak in chromatograms of both replicates for control and *aroD* respectively. Numbers on chromatogram indicate time of elusion in minutes of compound.

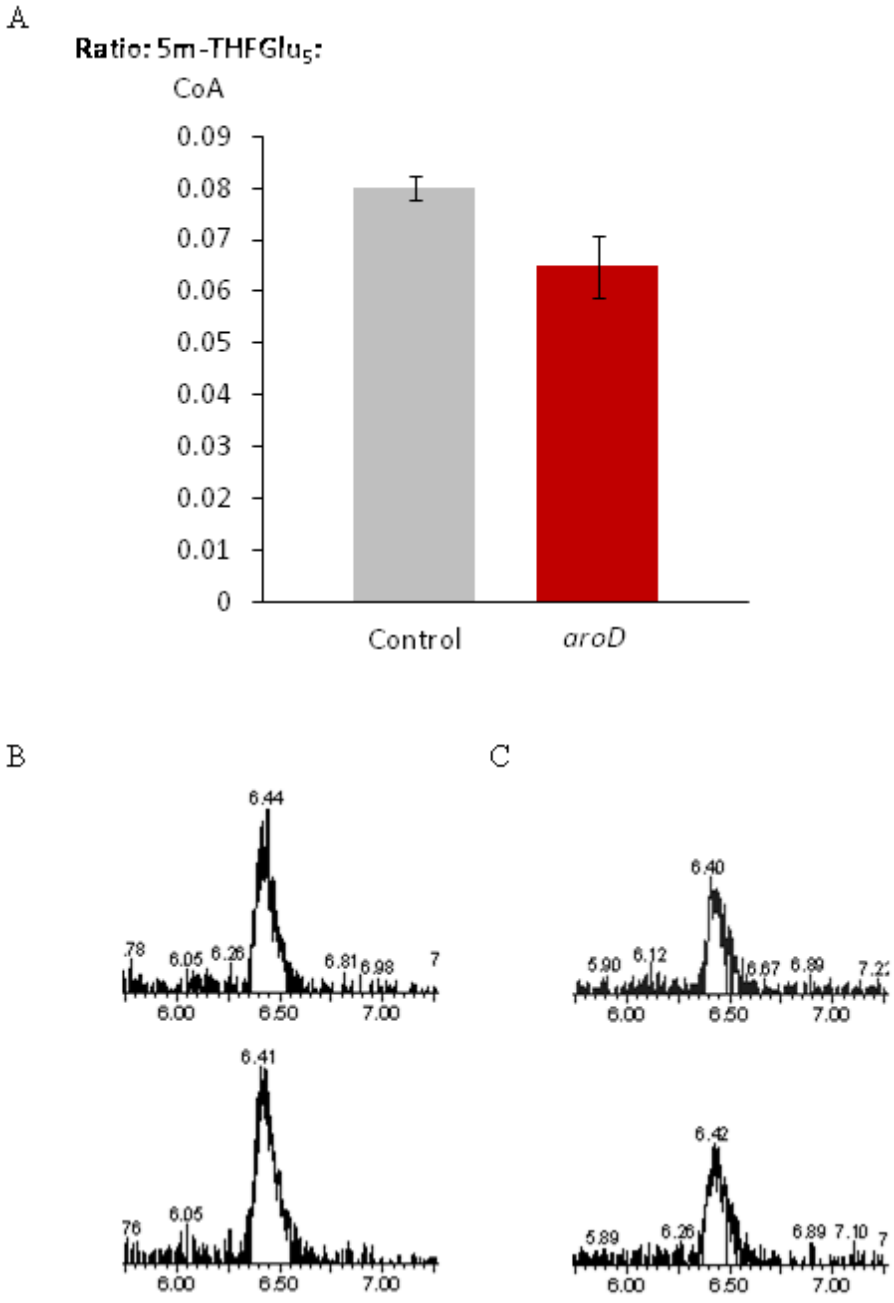


Figure 24: Levels of 5m-THFGlu$_5$ detected by LC-MS are decreased in *C. elegans* maintained on *aroD* mutant *E. coli* compared to *C. elegans* maintained on wild type *E. coli*. Data shown are from two biological replicates on high purity agar and peptone media. *C. elegans* strain used SS104. B) and C) show the 5m-THFGlu$_5$ peak in chromatograms of both replicates for animals maintained on control *E. coli* and *aroD* mutant *E. coli* respectively. Numbers on chromatogram indicate time of elusion in minutes of compound.

E. coli have much lower levels of folate than wild type *E. coli*, worms fed on *aroD E. coli* may be able to store or concentrate the folates obtained from the *E. coli*, as the level of 5m-THFGlu$_5$ shows only a small decrease when compared to those worms maintained on wild type *E. coli*, meaning that the level of folate required from *E. coli* by *C. elegans* is much lower than is available to them, i.e. *E. coli* exhibit excessive folate synthesis.

4.2.3 Folate analysis of SMX treated *E. coli*

To determine whether folate levels are decreased in SMX treated OP50 *E. coli*, a folate analysis was performed on OP50 *E. coli* (Control) and OP50 *E. coli* treated with 128 µg/ml SMX. Several folate species were detected, the most abundant of which was formyl-THFGlu$_3$ in concurrence with the most abundant folate in the wild type HT115(DE3) strain and the *aroD* mutant. Data show, the OP50 strain of *E. coli* has higher levels of formyl-THFGlu$_3$ than the HT115(DE3) wild type strain, respectively (Figure 23 and 25). The data show that SMX-treated *E. coli* show undetectable folate levels compared to OP50 control (Figure 25).

4.2.4 Folate analysis of *C. elegans* maintained on SMX treated *E. coli*

Extraction and analysis of worm folates was performed on *C. elegans* maintained on OP50, and on worms maintained on OP50 treated with 128 µg/ml SMX. As before, several folate species were detectable, the most abundant folate in these samples was 5m-THFGlu$_5$ in concurrence with data from worms maintained on HT115(DE3) *E. coli*. Results show that *C. elegans* maintained on OP50, show similar levels of 5m-THFGlu$_5$ as worms maintained on the wild type HT115(DE3) *E. coli* strain. (Figure 2 and 4). The levels of 5m-THFGlu$_5$ observed in *C. elegans* maintained on 128 µg/ml

SMX treated *E. coli*, was significantly lower than in *C. elegans* fed on OP50 (control), but detectable unlike the folate levels in *E. coli* treated with SMX (Figure 26).

The data from the SMX extractions show the same trend as the *aroD* extractions. In SMX treated *E. coli*, the decrease in folates compared to the control is much greater than in *C. elegans* maintained on SMX treated *E. coli*, compared to *C. elegans* fed on OP50. This data provide further evidence to support the hypothesis that *C. elegans* are able to store or concentrate the folate obtained from microbes, or obtain folate from the medium

This study shows that SMX lowers the level of the most abundant folates more dramatically in *E. coli* than the *aroD* mutation. This is perhaps expected as *aroD* is further upstream, in the shikimic acid pathway than dihydropteroate synthase (DHPS) the enzyme that SMX competes with para-aminobenzoic acid (PABA) for to inhibit folate synthesis. This suggests that in the *aroD* mutant *E. coli*, there may be compensation further downstream in the shikimic acid pathway. For example, small amounts of PABA may be obtained by the *E. coli* from the medium. This small amount of PABA may then be fed in to the folate cycle in the *aroD* mutant *E. coli* which would then help to partially restore the folate level to that of the wild type *E. coli*. SMX however, competes with PABA for DHPS, inhibiting DHPS from aiding the reaction in which PABA condenses with dihydropteroate pyrophosphate to produce the substrate needed to form dihydrofolate (DHF), as described in chapter 1 (section 1.9). Due to this specific targeting of folate synthesis, it is expected that SMX would decrease the folate levels of *E. coli* more severely than the *aroD* mutation.

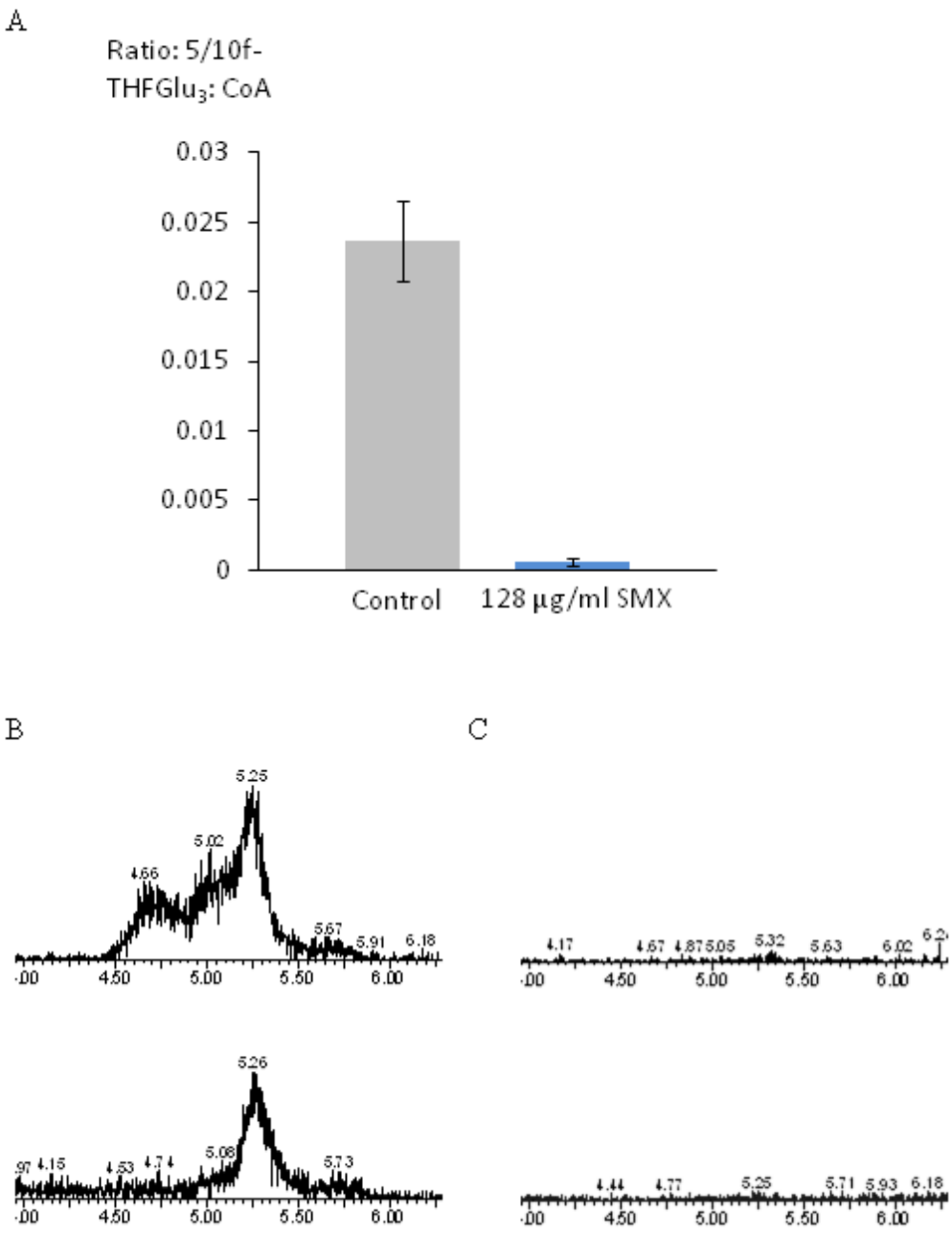


Figure 25: Levels of formyl-THFGlu$_3$ detected by LC-MS are decreased in SMX-treated OP50 *E. coli*. Data shown are from two biological replicates on high purity agar and peptone media. B) and C) show the formyl-THFGlu$_3$ peak in chromatograms of both replicates for control and *E. coli* treated with 128 µg/ml SMX respectively. The level of formyl-THFGlu$_3$ is beyond the limit of detection in SMX treated *E. coli*. Numbers on chromatogram indicate time of elusion in minutes of compound.

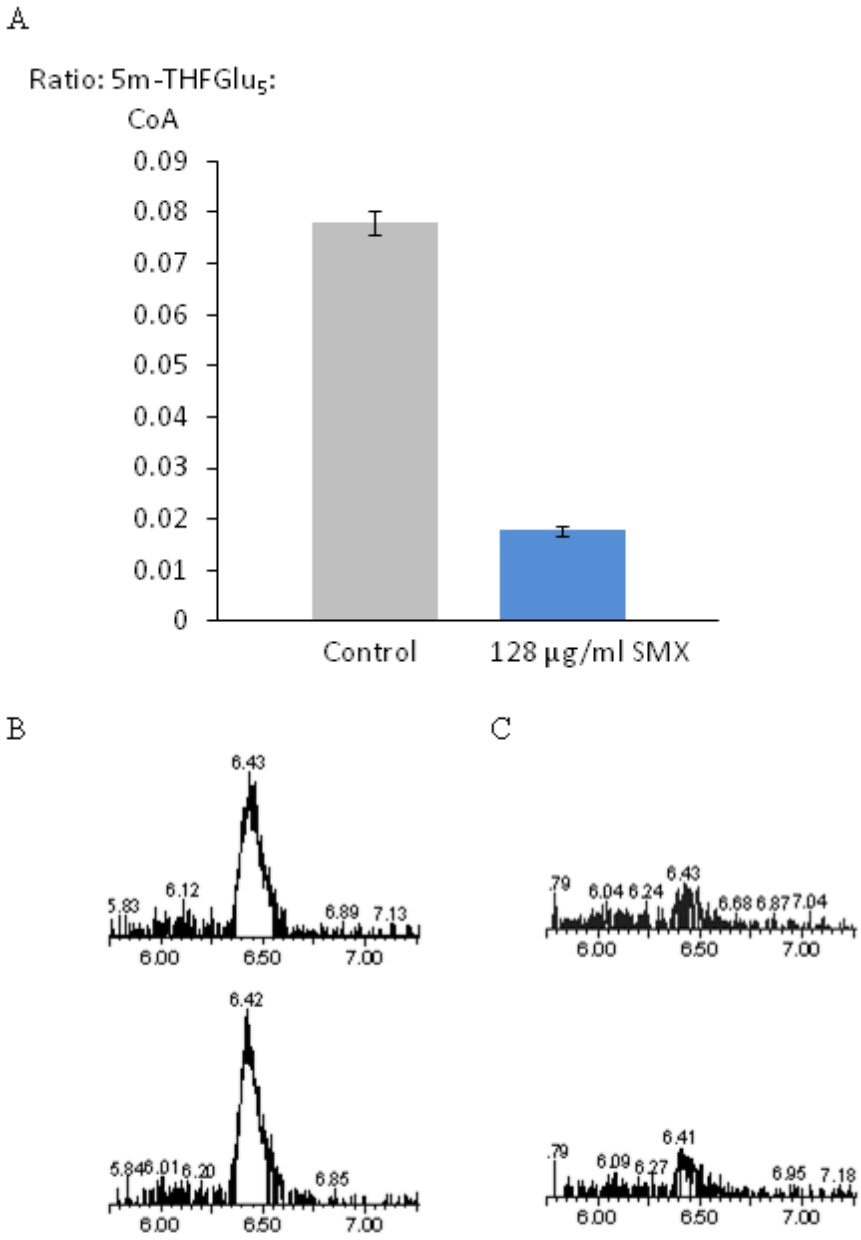


Figure 26: Levels of 5m-THFGlu$_5$ detected by LC-MS are decreased in *C. elegans* maintained on SMX treated OP50 *E. coli*. Data shown are from two biological replicates on high purity agar and peptone media. *C. elegans* strain used SS104 B) and C) show the 5m-THFGlu$_5$ peak in chromatograms of both replicates for animals maintained on control (OP50) *E. coli* and OP50 *E. coli* treated with 128 µg/ml SMX respectively. Numbers on chromatogram indicate time of elusion in minutes of compound.

4.2.5 SMX supplementation causes a dose dependent decrease in *E. coli* folate levels

Previous lifespan data as described in chapter 3 show that SMX causes a dose dependent increase in lifespan, with concentrations of 2 μg/ml SMX and above showing a significant increase in lifespan compared to the control. To determine whether this increase in lifespan correlated to a dose dependent decrease in folate levels, *E. coli* folate extractions and folate analysis were performed on *E. coli* that had been treated with concentrations of SMX ranging from 0.1 μg/ml – 128 μg/ml SMX. The results show that SMX causes a dose dependent decrease in the levels of formyl-$THFGlu_3$ until a concentration of 2 μg/ml SMX (Figure 27). In this particular experiment, from treatments of 2 μg/ml SMX upwards, the levels of formyl- $THFGlu_3$ became so decreased, that reliable analysis could not be performed. Thus the folate levels of SMX concentrations above 1 μg/ml were noted as below the limit of detection.

4.2.6 SMX supplementation causes a dose dependent decrease in *C. elegans* folate levels

This result coincides with the lifespan data showing that concentrations of SMX from 2 μg/ml and above, show a significant increase in *C. elegans* lifespan, suggesting that the lifespan increase observed is caused by a change in folate status of the *E. coli*. Extraction and analysis of folates was performed on *C. elegans* that had been maintained on OP50 treated with 0, 1, 4, 16 or 128 μg/ml SMX. Data from this study show that at concentrations of SMX above 1 μg/ml the levels of 5m-$THFGlu_5$ are severely decreased (Figure 28). In fact, in this instance the levels of 5m-$THFGlu_5$ in worms maintained on OP50 treated with 4, 16 or 128 μg/ml are not significantly

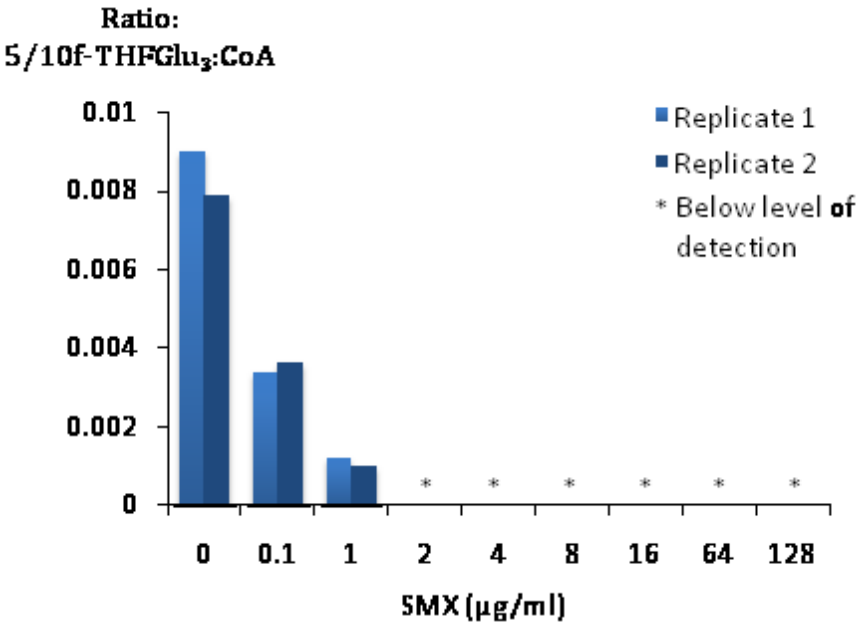


Figure 27: SMX supplementation causes a dose dependent decrease in *E. coli* OP50 levels of formyl THFGlu$_3$ as detected by LC-MS. Data from two biological replicates shown on high purity agar and peptone media. At 2 µg/ml SMX treatment levels of formyl THFGlu$_3$ become undetectable.

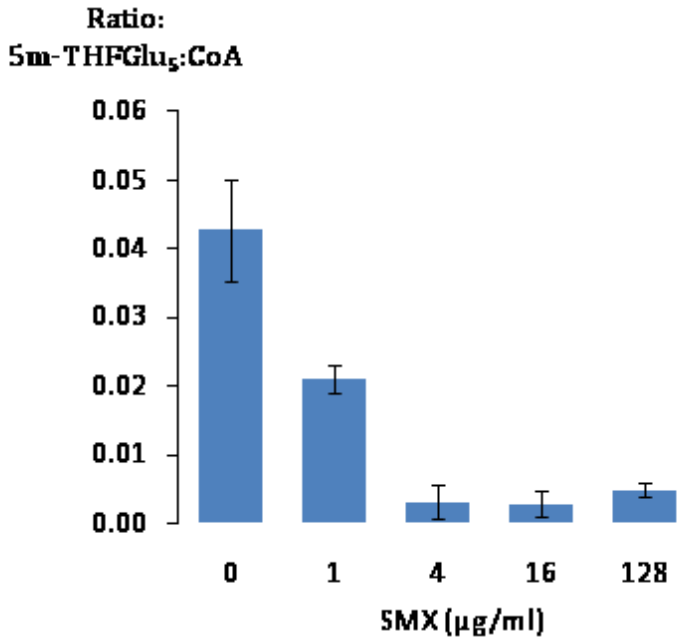


Figure 28: SMX supplementation causes a dose dependent decrease in *C. elegans* 5m-THFGlu$_5$ levels as detected by LC-MS. Data shown are from three biological replicates on high purity agar and peptone media. *C. elegans* strain used SS104.

different from each other. This data concurs with the *E. coli* extractions mentioned above and the lifespan data from chapter 3, suggesting that the lifespan extension observed with SMX treatment correlated to a decrease in *E. coli* folate levels.

4.2.7 Effects of low folate on development and fecundity of *C. elegans*

Having confirmed that SMX treatment significantly decreases folate levels in the *E. coli* and that *C. elegans* gets its folates from *E. coli,* I decided to investigate whether there were any negative side effects, caused to either the worm or *E. coli*, by these severely decreased folate levels. Folates are essential for nucleic acid synthesis, aromatic amino acid synthesis and other one-carbon metabolism including methylation and thus for healthy development and growth. A fecundity assay was performed on N2 (wild type) *C. elegans*, maintained on plates that had been supplemented with either 0, 16 or 128 μg/ml SMX. In this assay single worms were maintained individually on plates, transferred every 24 hours to fresh plates and the number of eggs layed each day was counted. Data from this assay show that SMX treatment does not alter the time taken for *C. elegans* to reach the egg laying stage of adulthood. Treatment with either 16 or 128 μg/ml SMX does not affect the egg laying cycle; eggs are layed at the same time and rate as worms maintained on plates with no SMX supplementation (Figure 29a). Treatment with SMX does not have any significant effect on total brood size (Figure 29b), however it is important to note that the number of eggs layed in self fertilised worms is limited by sperm number. Therefore, even though SMX treatment significantly decreases the level of folate in *C. elegans*, worms maintained on SMX treated *E. coli*, still have sufficient folate levels for normal growth and reproduction.

4.2.8 Effects of low folate on worm motility

To further investigate whether SMX might be having any deleterious effects on *C. elegans*, a motility assay was performed on worms maintained on plates with either 0, 16 or 128 μg/ml SMX supplementation. In this assay I am using motility as a biomarker of health. The scoring of motility was performed based on an A,B, C class system used by Herndon et al, where animals in class A move constantly leaving sinusoidal tracks, animals in class B do not move unless prodded and animals in class C exhibit a head or tail twitch when prodded (Herndon et al. 2002). All animals begin adulthood with class A motility. Data from this assay show that worms with no SMX supplementation remained in class A motility for an average of 8.4 days. *C. elegans* that were kept on plates with 16 μg/ml SMX supplementation showed class A motility for significantly longer into adulthood, with a mean of 11.3 days ($P<0.0001$). *C. elegans* maintained on plates with 128 μg/ml SMX treatment showed the longest period with class A motility, an average of 13.2 days ($P<0.0001$) (Figure 30). These data show that SMX does not appear to have any negative effects on worm motility, in fact worms treated with SMX remain mobile for significantly longer, suggesting that SMX slows ageing.

As SMX supplementation does not appear to have any side effects on the worms, and given that the decrease in folate levels is greater in *E. coli*, we decided to investigate *E. coli* growth rates with regard to SMX treatment. SMX is a bacteriostatic antibiotic, so it would be expected that the drug prevents bacterial proliferation. If this was the case then it may not be so surprising that the worms maintained on such treated *E. coli*

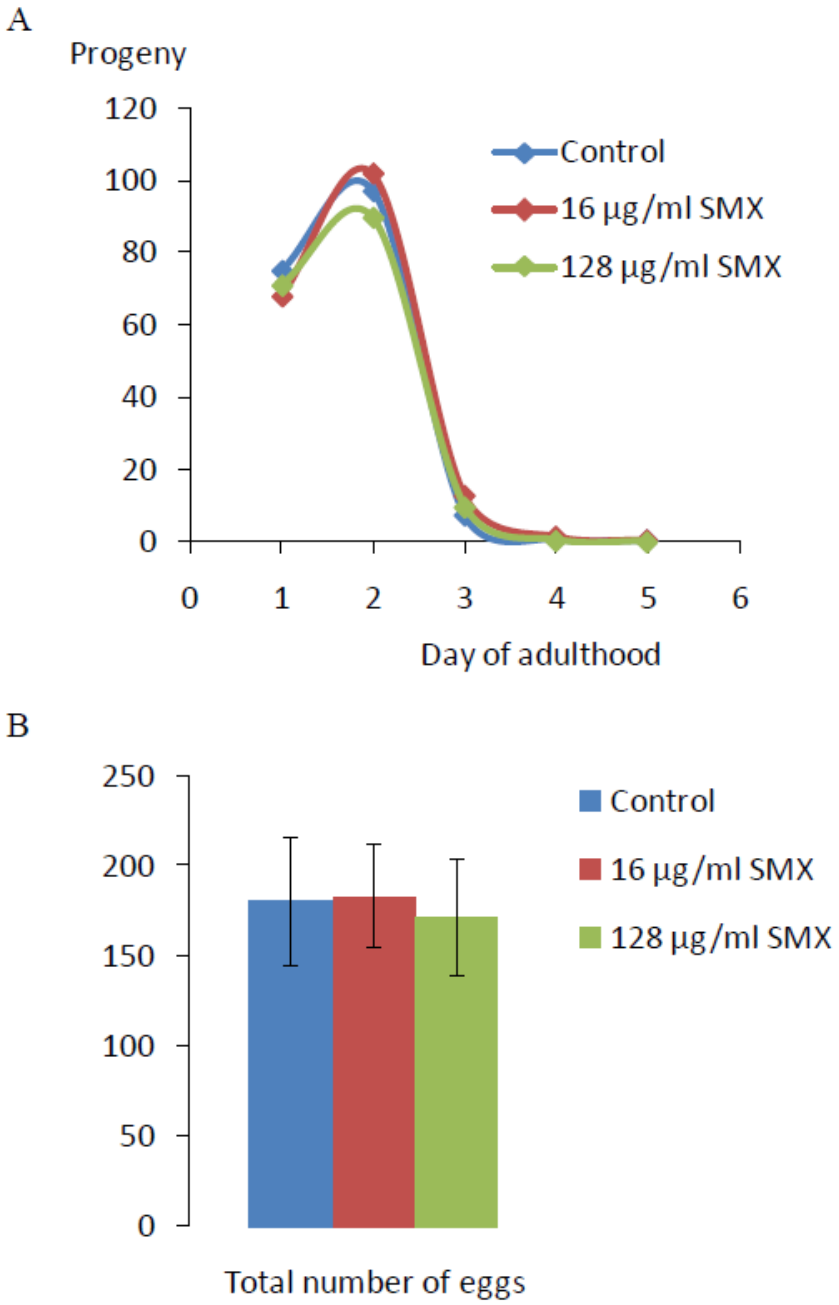


Figure 29: SMX appears to have no effect on A) development time, egg-laying schedule or B) total brood size of N2 worms raised at 25°C. Experiment performed on high purity agar and peptone media. *E. coli* strain used OP50. Control (number of animals = 15), 16 µg/ml SMX (n = 18), 128 µg/ml SMX (n = 17). Error bars are ± standard deviation

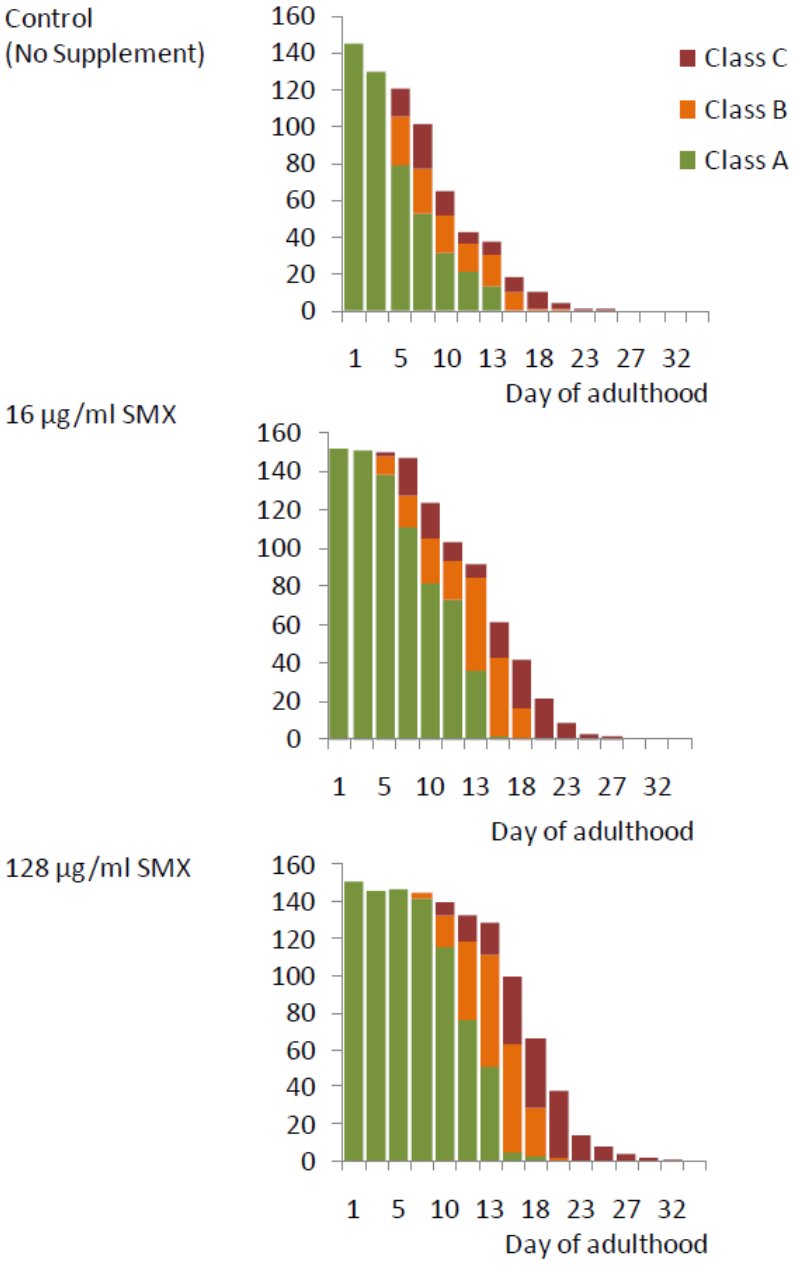


Figure 30: *C. elegans* maintained on SMX-treated *E. coli* show Class A motility for longer throughout adulthood. Experiment performed on high purity agar and peptone media. *C. elegans* strain used SS104. *E. coli* strain used OP50. Control n = 130 mean number of days in class A motility = 8.40 days, 16 µg/ml SMX n = 151 mean number of days in class A motility = 11.34 days, 128 µg/ml SMX n = 145 mean number of days in class A motility = 13.21 days.

would live longer, due to dietary restriction effects or pathogenic effects from proliferation.

4.2.9 Effects of SMX treatment on *E. coli* growth

Growth of *E. coli* both in liquid culture and on NGM plates was investigated. Data show that in liquid culture, neither 16 μg/ml SMX nor 128 μg/ml the highest dose of SMX used for lifespan extension, significantly affect *E. coli* proliferation (Figure 31). Results from bacterial lawn density on NGM plates, show that SMX has minimal effect on *E. coli* proliferation on agar plates (Control vs. 128 μg/ml SMX P =0.03) (Figure 32). To ensure that the *E. coli* observed in this proliferation assay were viable, colony forming unit assays were performed on bacterial lawns from plates with no

SMX supplementation, and bacterial lawns from plates with 128 μg/ml SMX supplementation. Kanamycin treatment was used as a negative control due to its anti-proliferative capacity. Data show that there was no significant difference between OP50 proliferation and 128 μg/ml SMX treated OP50 proliferation, confirming that the *E. coli* were proliferating and viable (Figure 33). This result suggests that the relatively low dose of SMX used, does not have any apparent negative side effects on *E. coli* growth, in spite of lowering the levels of the most abundant folates dramatically.

4.3 Discussion

In this chapter I have quantified the levels of the most abundant folate - formyl-THFGlu$_3$ - in the *aroD* mutant and *E. coli* treated with SMX compared to the

appropriate controls. The *E. coli* folate extraction method, has been modified and

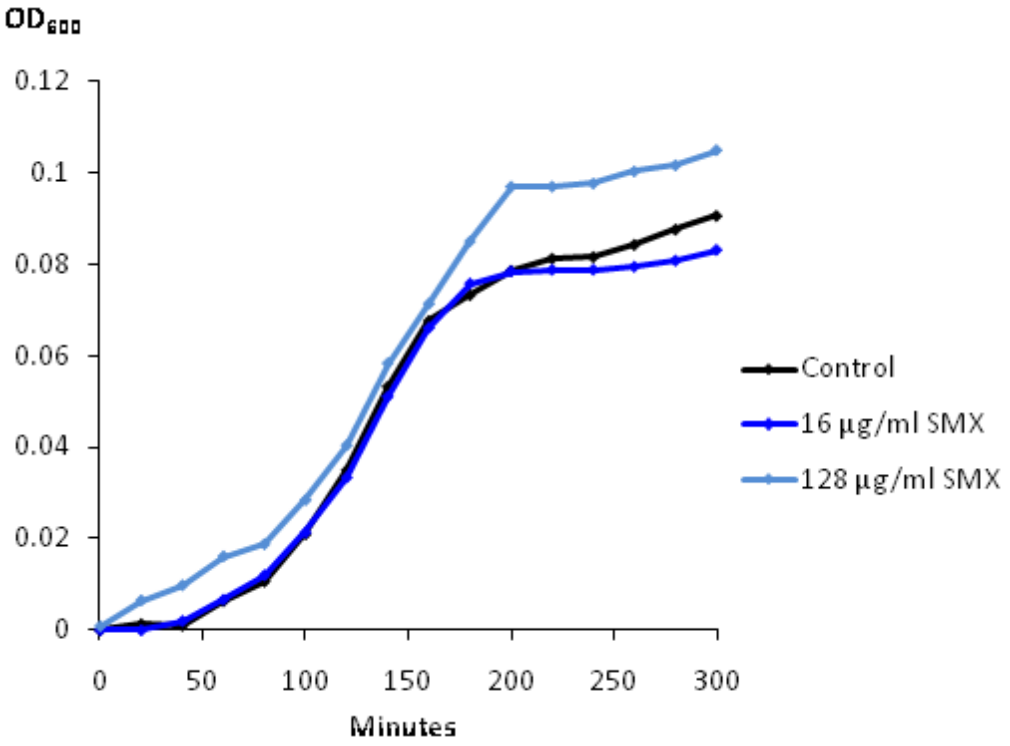


Figure 31: SMX shows no significant negative effect on OP50 growth in liquid culture. Experiment performed in liquid peptone media. *E. coli* strain used is OP50. OD_{600} measurements of bacterial density taken every 20 minutes show that SMX supplementation at 16 and 128 µg/ml has no effect on the log-phase growth rate of OP50 in liquid NGM at 37°C. Data shown are mean values from 3 biological replicates

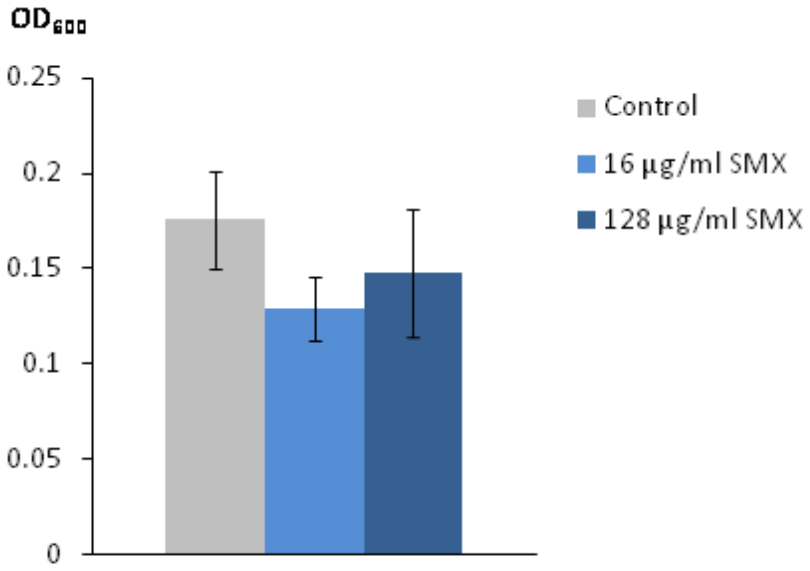
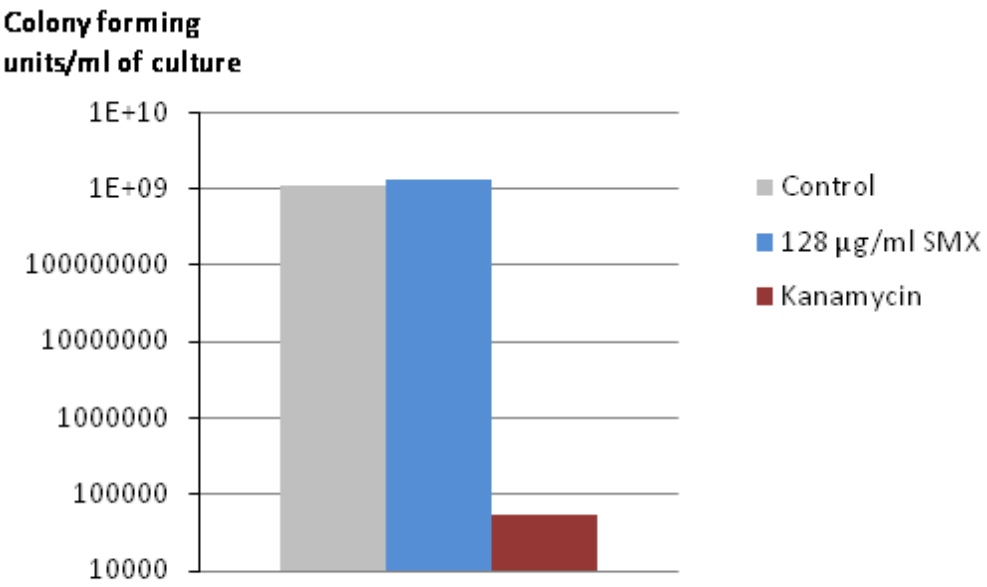


Figure 32: SMX shows minimal negative effect on OP50 growth on NGM high purity agar plates. OD_{600} measurements of relative bacterial content of established lawns from the mean values of 13 plates per condition. Error bars are ± standard deviation. Student's t-test values (Control vs. 16 µg/ml SMX: P<0.0001, Control vs. 128 µg/ml SMX: P=0.032, 16 µg/ml SMX vs. 128 µg/ml SMX: (n.s, P = 0.091)).



Dilution	Replicate	Control	SMX	Kanamycin
10^{-2}	1	Lawn	Lawn	53
	2	Lawn	Lawn	61
	3	Lawn	Lawn	50
10^{-4}	1	$>10^5$	$>10^5$	4
	2	$>10^5$	$>10^5$	4
	3	$>10^5$	$>10^5$	6
10^{-6}	1	87	105	2
	2	117	147	2
	3	118	132	1
10^{-7}	1	10	15	0
	2	9	18	1
	3	7	18	1
10^{-8}	1	2	3	0
	2	2	2	0
	3	0	3	0

Figure 33: SMX-treated OP50 show no significant difference in the number of colony forming units (CFU) per ml of culture compared to control. Colony forming units from bacteria scraped from established lawns on high purity agar plates. Student's t-test values (Control vs. 128 µg/ml SMX: n.s, P = 0.26).

optimised for use in extraction of *C. elegans* folates, which has not been carried out previously to the best of our knowledge. Following the optimisation process, levels of the most abundant folate in worms - 5m-THFGlu$_5$ – have been quantified in *C. elegans* maintained on *aroD E. coli*, or maintained on SMX treated *E. coli* and compared to the relative controls. These data have shown that SMX is a more effective intervention to inhibiting the folate cycle than the *aroD* mutation is, as it decreases the folate levels in both SMX treated *E. coli*, and worms kept on SMX treated *E. coli* more dramatically.

Despite the observed decrease in folate levels, observed with SMX supplementation, *C. elegans* development, reproductive cycle or total brood size was not affected. However, it remains to be determined whether continued SMX treatment, and therefore low folates, had any long term effects. SMX treatment shows a minimal effect on *E. coli* growth, suggesting that SMX is not working via dietary restriction. *C. elegans* appear to have more motility for a longer period on SMX supplemented plates, implying that the animals not only live longer but also have an increase health span.

Having confirmed and quantified the folate levels with SMX treatment, we can be confident that SMX causes increased *C. elegans* longevity in a folate dependent manner. Levels of the most abundant folates in both worms and *E. coli* treated with SMX supplementation are significantly decreased compared to the controls, which might make us question how the *E. coli* and worms are able to grow and develop normally, with such low folate levels.

The LC-MS method of detection that we are employing, is able to detect a number of folate species. However it is not sensitive enough to be able to detect and quantify all folate species present. We are using the most abundant folates, 5m-THFGlu_5 and formyl- THFGlu_3 in worms and *E. coli* respectively for quantification, to enable us to determine whether folate levels are decreased with SMX supplementation. It may be possible that while levels of these abundant folates are largely decreased, other folate species that we are unable to quantify, are not as dramatically affected. To obtain a more complete picture of the level of folate inhibition, and perhaps more insight into the pathway via which SMX is causing an increase in lifespan, a more sensitive detection method would be required.

In this chapter I have shown that increase in lifespan caused by SMX correlates to a decrease in the levels of the most abundant folates. However, whilst this suggests that SMX is causing the extension in *C. elegans* longevity in a folate dependent manner, further work is required to uncover the specific mechanism via which SMX is working.

CHAPTER 5

THE ROLE OF ANIMAL FOLATES IN LIFESPAN DETERMINATION

5.1 Introduction

5.1.2 Methotrexate

The drug methotrexate (MTX) was developed in the 1940's as an antagonist of folic cycle in animals/humans. At the time of its synthesis it was a unique compound in that it was a tightly-binding enzyme inhibitor with a specific target (Chan & Cronstein 2002; Chabner et al. 1985). MTX inhibits the enzyme dihydrofolate reductase (DHFR), which is essential in the animal folate cycle, to convert dietary folate, DHF into THF which is the more biologically useful form of folate. THF is the precursor of the active folate forms that are required for synthesis of purines, methionine and serine (Chabner et al. 1985). MTX was initially employed as an anti proliferative agent for tumour cells, that inhibits purines and pyrimidines for the therapy of malignancies, but is now used also for inflammatory diseases such as rheumatoid arthritis (Whittle & Hughes 2004; Chan & Cronstein 2002; Chabner et al. 1985). It has been shown that 100 μg/ml MTX has no effect on wild type animals, but causes *nuc-1* worms to become sterile, slow developing and uncoordinated (Mello et al. 1991). The *nuc-1* worms lack the *nuc-1* nuclease that usually derives purines from the digestion of bacteria DNA in the worm gut (Lyon et al. 2000). In normal conditions the folate cycle in *nuc-1* animals would synthesise the required purines. In the presence of MTX however, the folate cycle is also inhibited resulting in the slow growth observed in *nuc-1* mutants.

Having shown that SMX causes its lifespan increase in a folate dependent manner, it still remains unclear whether SMX is causing lifespan extension by a direct effect on the animal itself, or inhibition of excess folate synthesis in *E. coli* causing metabolic change, and inhibiting a life shortening function in the *E. coli*. Data presented in chapter ,3 regarding sulfonamide resistant *E. coli* suggest that the lifespan increase is due to SMX treatment of the *E. coli*, as when worms were maintained on SMX treated *E. coli* possessing the R26 plasmid which confers resistance to SMX, no lifespan increase in *C. elegans* was observed.

5.1.3 *folt-1* and *gcp-2.1 C. elegans* mutants

One of the many advantages of *C. elegans* as a model system is the availability of deletion mutants. A review of the literature and knockout collections flagged folate related worm mutants such as *folt-1* and *gcp-2.1*. These mutant worms available from the CGC, might enable us to manipulate animal folates independently of *E. coli* folate. As levels of folate are much higher than required for growth in *C. elegans* maintained on *E. coli* under normal circumstances, it may be possible that reducing the amount of excess folate in the animal, may increase animal lifespan.

Folt-1 is one of 3 folate transporters (*folt-1, folt-2* and *folt-3*) in *C. elegans* characterised by Balamuragan et al (Balamurugan et al. 2007; Austin et al. 2010). The *folt-1*homozygous is lethal sterile, and therefore the strain must be maintained as heterozygotes. In order for maintenance of this strain a balancer chromosome is used, the balancer chromosome is marked with GFP. In *folt*-1 heterozygous worms the GFP is expressed in the pharynx. When *folt-1* heterozygotes individuals segregate

heterozygous wild type GFP worms, arrested nT1aneuploids (as individuals that are homozygous for the balancer chromosome are not viable), and non-GFP homozygous *folt-1* mutants (Balamurugan et al. 2007). The strain is readily available from the CGC.

Glutamate carboxypeptidase II (GCPII) is an enzyme initially characterized in mammals, required to remove glutamate moieties from polyglutamated dietary folate. Removal of glutamate molecules, allows uptake of folate species by folate transporters that preferentially import monoglutamated folate molecules (Halsted 1998). There are three homologues of GCPII in *C. elegans*, encoded by R57.1 (named *gcp-2.1*), C35C5.2 (named *gcp-2.2*) and C35C5.11 (named *gcp-2.3*). *gcp-2.1*, *gcp-2.2*, and *gcp-2.3* were named by our group. A review of the literature and my own data from mass spectrometry suggests that *E. coli* contain mainly polyglutamated folates (Kwon et al. 2008b; Lu et al. 2007), implying the need for a GCPII in *C. elegans* to allow efficient folate uptake. A deletion of the *gcp-2.1* gene was available from the CGC.

5.1.4 Aims and Objectives

In this chapter I aim to look further into the mechanism via which sulfamethoxazole (SMX) works to increase animal lifespan. Using folate specific *C. elegans* mutants and other chemical interventions such as MTX, I try to decipher and understand the specific pathway within which SMX acts in a folate dependent fashion to extend animal lifespan.

5.2 Results

5.2.1 Methotrexate and SMX development assay

A development assay was performed to determine whether MTX would induce slow growth and sterility in worms fed on SMX treated OP50 *E. coli*. The data show that whilst N2 strain *C. elegans* develop to adulthood normally under all conditions, adults appear to produce dead eggs with an apparent decrease in total number of eggs layed as MTX concentration is increased in the presence of SMX supplementation. Representative images of these observations are shown in Figure 34. This effect has been observed to become progressively more pronounced as the dose of MTX is increased (from 100 μg/ml to 800 μg/ml). To determine whether inhibition of the worm folate cycle using MTX supplementation increased lifespan in a similar manner to SMX, lifespan analysis was carried out on *C. elegans* maintained on OP50 *E. coli* treated with 100 μg/ml MTX, 128 μg/ml SMX, or a combination of both drugs. Worms maintained on MTX treated *E. coli* did not show extended longevity. MTX supplementation did not further extend the longevity observed in worms maintained on SMX treated *E. coli* (Figure 35). These data suggest that inhibiting the *C. elegans* folate cycle does not extend lifespan. In order to be certain that the *C. elegans* folate cycle is inhibited by MTX at this concentration, folate levels in *C. elegans* maintained on MTX treated OP50 would need to be confirmed by liquid chromatography coupled to mass spectrometry (LC-MS).

5.2.2 *folt-1 C .elegans* mutant

The *C. elegans* mutant *folt-1* possesses a deletion in a folate transporter, resulting in inhibited folate uptake. As obtained from the CGC, this mutant could not be used for

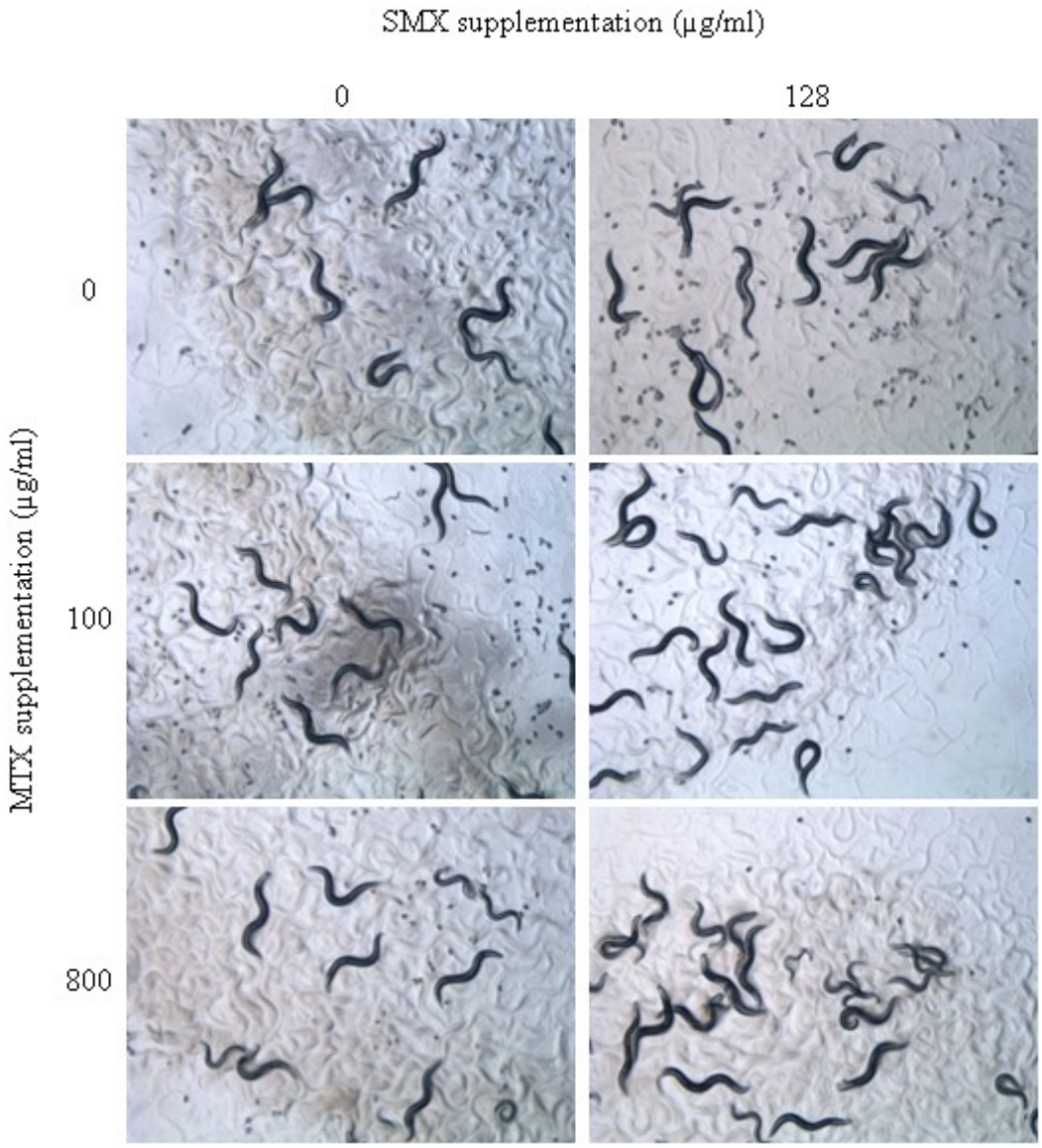


Figure 34: Developmental assay showing *C. elegans* maintained on *E. coli* treated with both SMX and MTX. High purity and peptone media used. *C. elegans* strains used N2, *E. coli* strain used OP50. Images taken on day 4 of adulthood. 3 biological replicates were performed for each condition, with a representative image shown here. Development at 25 °C. On further investigation it was observed that when MTX and SMX are combined the eggs layed are dead and do not hatch.

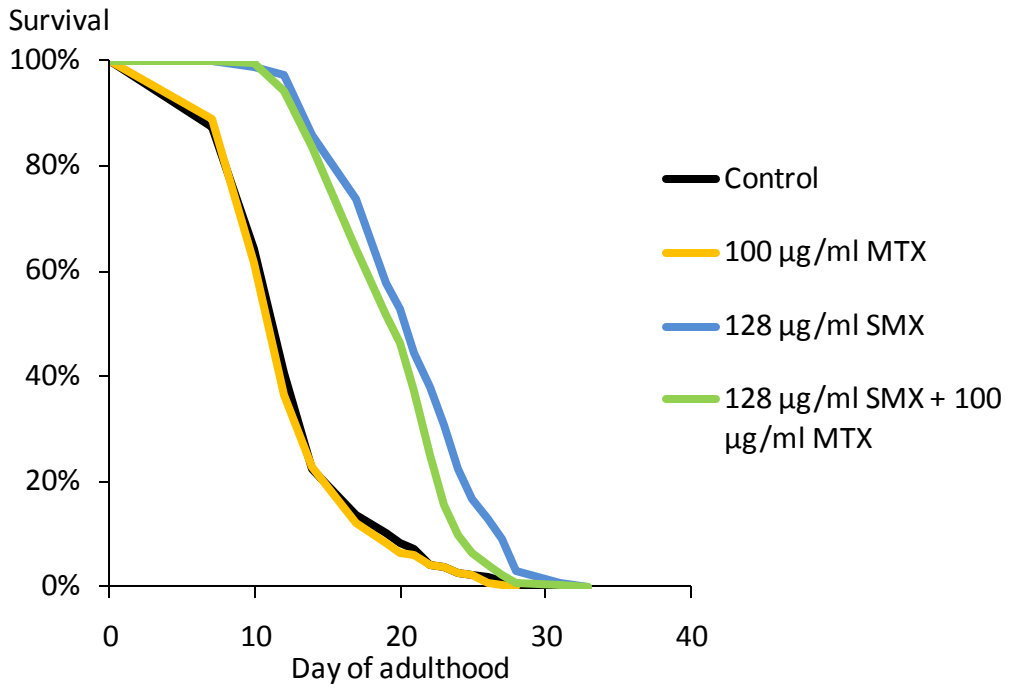


Figure 35: MTX does not significantly increase *C. elegans* lifespan. Lifespan analysis performed on peptone high purity agar. *C. elegans* strains used SS104, *E. coli* strain used OP50. Control n = 303 mean lifespan = 13.09 days, 100 µg/ml MTX n = 325 mean lifespan = 12.90 days (n.s), 128 µg/ml SMX n = 338 mean lifespan = 20.92 days ($P<0.0001$), 128 µg/ml SMX + 100 µg/ml MTX n = 309 mean lifespan = 19.68 days ($P<0.0001$). Data shown representative of from two biological replicates.

lifespan analysis, as adult homozygous *folt-1* individuals were sterile and short lived (Balamurugan et al. 2007). Many of the mutated worm strains have not been outcrossed from the CGC. However outcrossing is important as worm mutants are produced using potent mutagens, and therefore it is possible for many mutations to occur. Outcrossing limits the number of background mutations present. Therefore we attempted to outcross the *folt-1* mutant to the N2 wild type *C. elegans* strain, in order to produce a fertile mutant that could be used for lifespan analysis. I hypothesised that sterility might be a linked mutation to *folt-1*. Single worm PCR of 96 worms, identified 23 homozygous *folt-1* worms, however all 23 were sterile. This result confirmed that the sterility phenotype was closely linked to the homozygous *folt-1* mutant, and could not be separated by recombination. (Figure 36).

5.2.3 *gcp-2.-1 C. elegans* mutant shows a growth defect on SMX

Therefore, we turned our attention to the *C. elegans gcp-2.1* mutant. In order to determine whether folate uptake was impeded in this mutant, a developmental assay was carried out. The *gcp-2.1* mutant animals were healthy and showed normal growth on control and 100 μg/ml MTX treated *E. coli*. Perhaps surprisingly, when compared to wild type animals, the *gcp-2.1* mutants appeared to show slow development, were uncoordinated and sterile on 128 μg/ml treated *E. coli*. Representative images of these observations are shown in Figure 37. This result is consistent with a lack of monoglutamated folate species available to the worm from the *E. coli*; however folate levels would need to be confirmed by LC-MS.

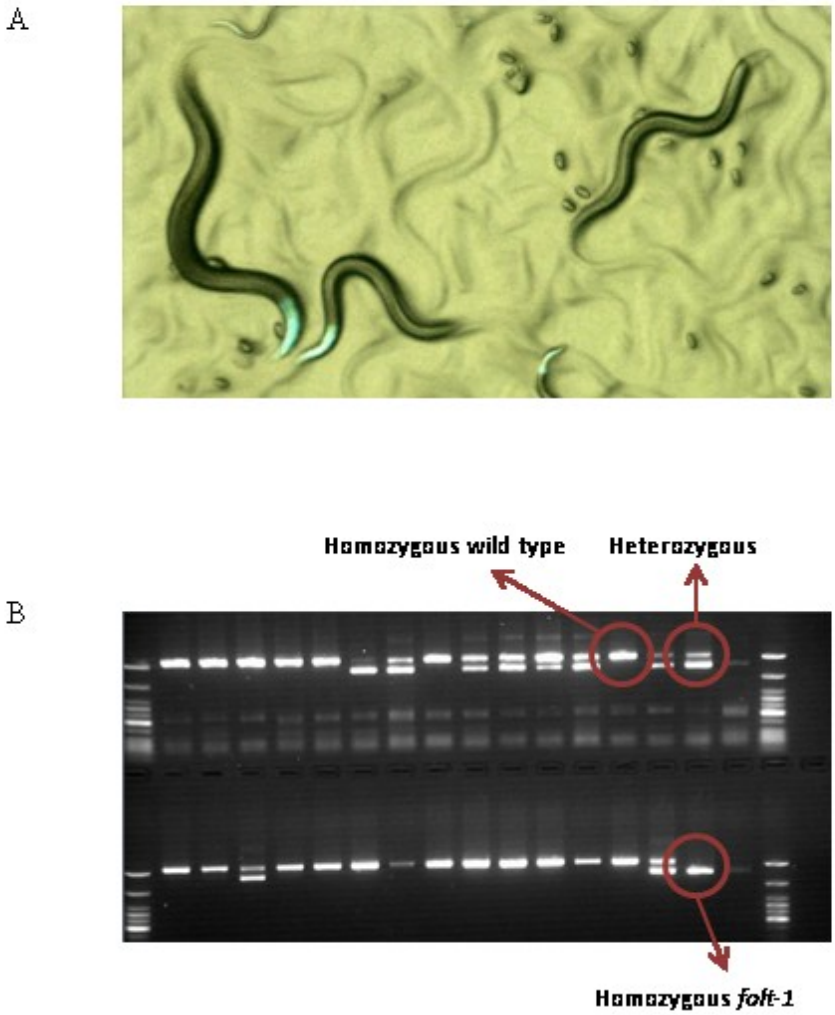


Figure 36: Outcross and genotyping of the *folt-1 C. elegans* mutant. A) showing the GFP tagged pharynx of 2 heterozygous *folt-1* individuals (left), and a non-GFP expressing *folt-1* homozygous individual (right). B) A representative agarose gel showing the *folt-1* genotype of 32 individual worms as a result of single worm PCR. Each lane represents a single worm. Presence of 2 bands indicates a heterozygote. Presence of lower band only indicates that the worm is homozygous for *folt-1*, presence of upper band only indicates homozygous wild type individual

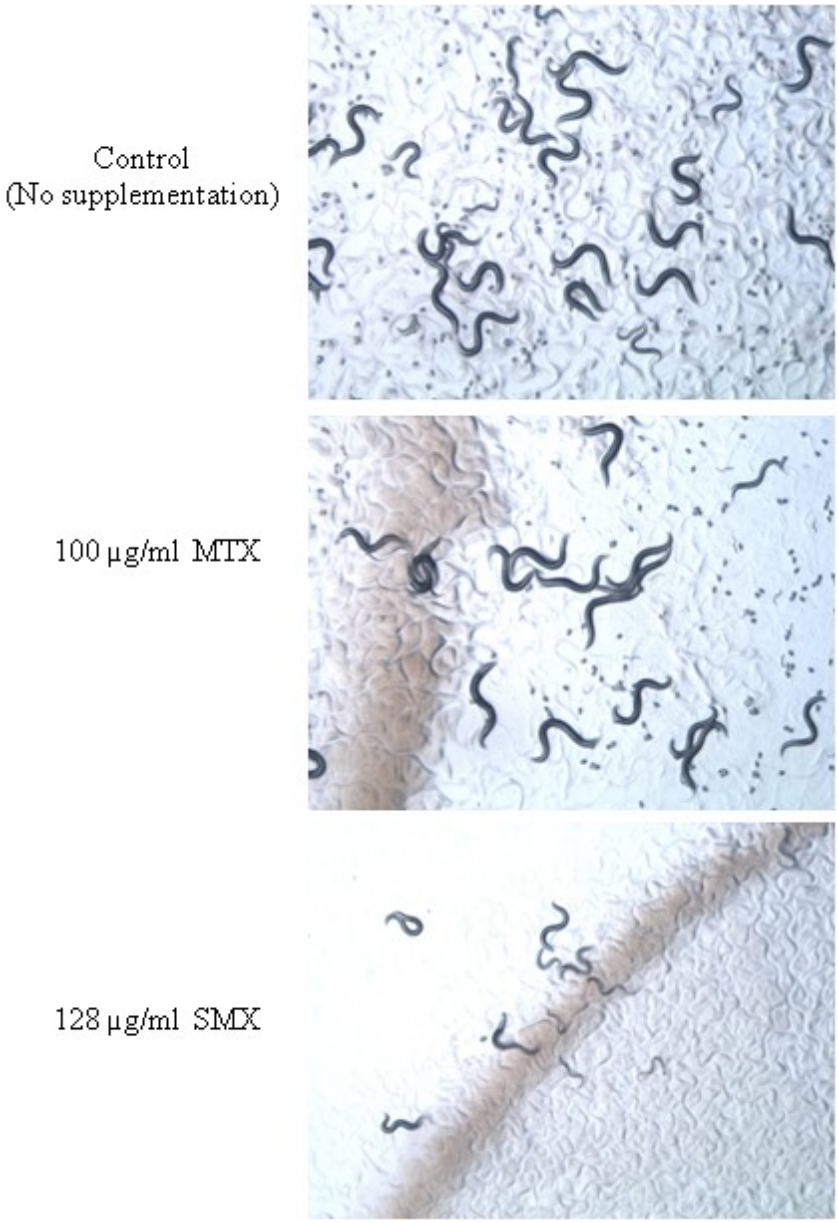


Figure 37: Developmental assay showing *gcp-2.1 C. elegans* maintained on OP50 *E. coli* treated with both SMX and MTX. High purity agar and peptone media used. Images taken on day 4 of adulthood. 3 biological replicates were performed for each condition, with a representative image shown here. Development at 25 °C

5.2.4 Lifespan analysis of *gcp-2.1*

Given the observed growth defect of the *gcp-2.1* mutant, we assume that the defect is caused by the level of useable folates in these worms being decreased. Preliminary lifespan analysis was performed to determine whether the decrease of monoglutamated folates correlates with an extension in longevity. Results of this study show that the *gcp-2.1* mutant is significantly longer lived (15.50 days) than the wild type control (13.52 days) ($P<0.0001$) (Figure 38). These preliminary data are consistent with the hypothesis that the inhibition of folate uptake in the animal, results in a significant increase of *C. elegans* longevity, folate measurement by LC-MS would provide confirmation of these data.

Whilst this data is consistent with our previous hypothesis that removal of excessive folate might increase animal lifespan, a review of the strain background showed that the mutant had not been outcrossed since being discovered. To confirm that the increase in lifespan observed was due to the *gcp-2.1* mutant and not perhaps an undetected mutation, a summer student, Marta Ciplinski, outcrossed the *gcp-2.1* mutant three times to wild type, and confirmed that the outcrossed *gcp-2.1* mutant exhibited the same growth defect when maintained on SMX supplemented plates. I then used strains of mutant *gcp-2.1* and wild type siblings isolated from the most recent outcross for further experimentation. A developmental assay was performed with the *gcp-2.1* mutant and the respective wild type *C. elegans* maintained on control *E. coli*, 100 μg/ml MTX treated *E. coli*, and 128 μg/ml SMX treated *E. coli*. Results were consistent with the previous findings, *gcp-2.1* mutant *C. elegans* showed normal development on control *E. coli*, and *E. coli* treated with 100μg/ml MTX. On *E. coli* that had been supplemented with 128 μg/ml SMX, *gcp-2.1* mutant *C. elegans*

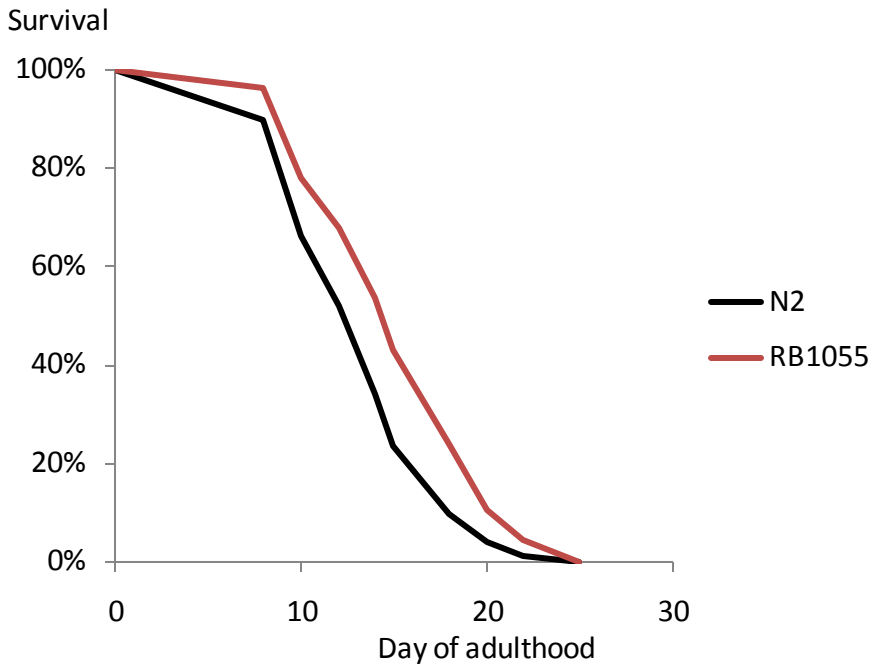


Figure 38: *gcp-2.1* mutant *C. elegans* show a significant increase in lifespan. Lifespan analysis performed on peptone high purity agar. *C. elegans* strain used RB1055 (*gcp-2.1*) and N2. *E. coli* strain used OP50. N2 n = 160 mean lifespan = 13.52days, RB1055 (*gcp-2.1*) n = 163 mean lifespan = 15.50 days ($P<0.0001$).

appeared to show sterility, uncoordinated movement and slow growth in accordance with previous findings. Representative images of these observations are shown in Figure 39. Sensitivity of the *gcp-2.1* mutant to SMX treated *E. coli* was rescued by a transgene, containing a genomic fragment, including a wild type copy of *gcp-2.1* gene made by Marta (data not shown). Therefore the growth defect observed is due to the *gcp-2.1* deletion, we assume that *gcp-2.1* decreases folate uptake in the animal, but this would have to be confirmed by measuring folate levels using liquid chromatography paired with mass spectrometry (LC-MS).

5.2.5 Lifespan analysis of outcrossed *gcp-2.1*

Lifespan analysis was performed with newly outcrossed *gcp-2.1* mutant and the respective wild type control. Before outcrossing, the *gcp-2.1* mutant had shown a significant increase in longevity compared to N2 wild type strains. After outcrossing, the *gcp-2.1* mutant lifespan can be compared against the lifespan of a wild type sibling, from the same genetic background. Results show that the *gcp-2.1* shows no significant increase in lifespan with regards to the control or the *gcp-2.1* transgenic strain (Figure 40). Furthermore, we can disregard our previous findings and suggest that inhibiting folate uptake in *C. elegans* does not result in extended longevity, as the outcrossed strain of *gcp-2.1* does not increase lifespan. Folate measurement by LC-MS would be required to confirm this hypothesis.

5.2.6 Folinic acid supplementation rescues the *gcp-2.1* growth defect

As shown, 10 μM folinic acid supplementation was able to fully rescue the growth defect observed in *gcp-2.1* mutant animals that had been maintained on SMX treated

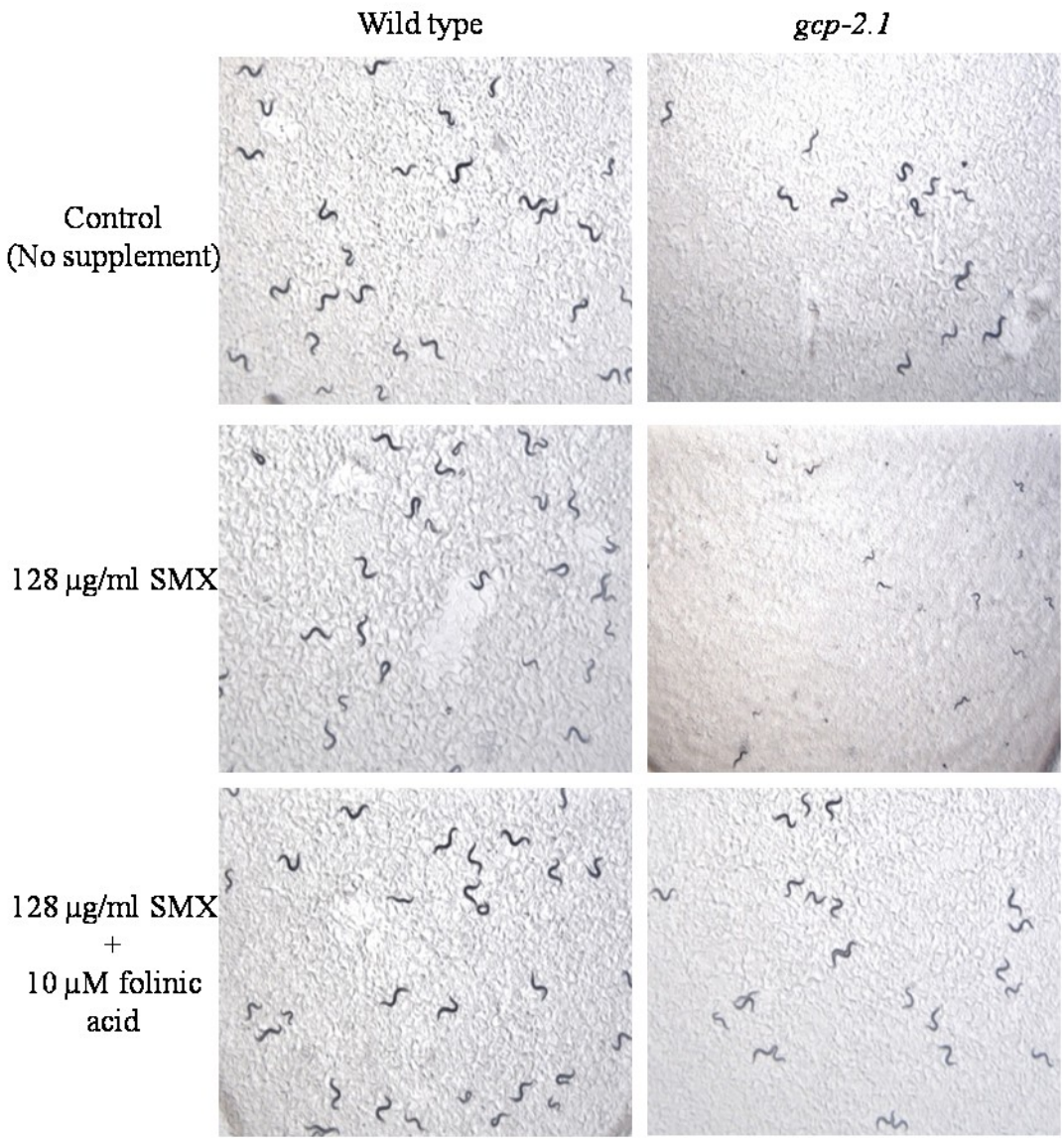


Figure 39: Developmental assay showing growth phenotype of the *gcp-2.1* mutant *C. elegans* maintained on SMX treated OP50 *E. coli*, and rescue with folinic acid. High purity agar and peptone media used. *C. elegans* strain used UF208 (Wild type) and UF209. Images taken on day 3 of control adulthood. 3 biological replicates were performed, representative images are shown.

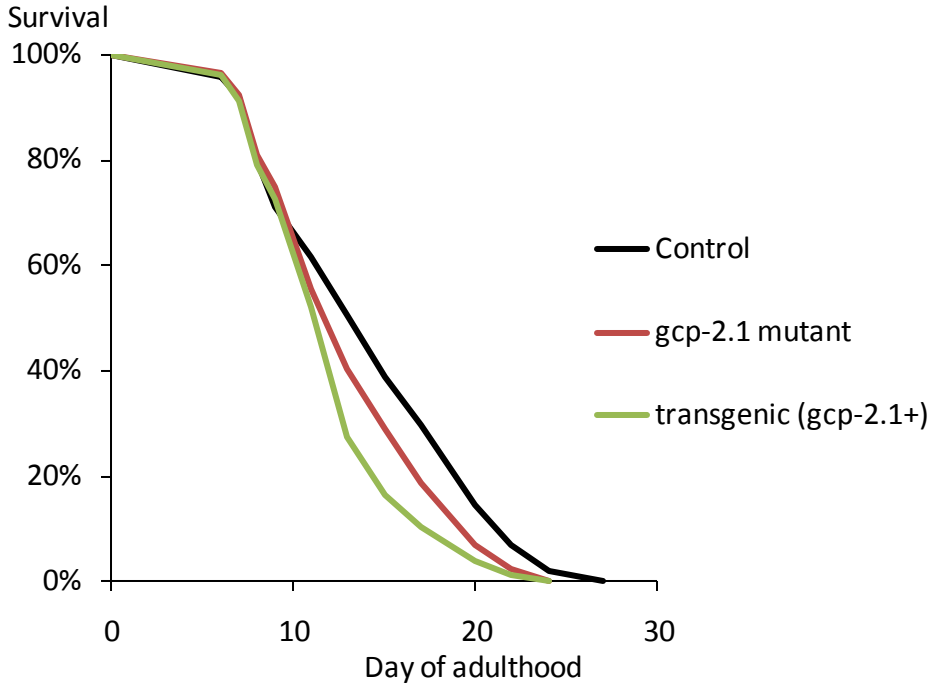


Figure 40: The *gcp-2.1* mutant is not significantly long lived. Lifespan analysis performed on peptone high purity agar. *C. elegans* strains used UF208, (wild type), UF209 (*gcp-2.1*) and UF215 (*gcp-2.1*+) Control n = 160 mean lifespan = 14.49 days, *gcp-2.1* mutant n = 167 mean lifespan = 13.44 days (n.s), transgenic (*gcp-2.1*+) n = 172 mean lifespan = 12.45 days (n.s). Data shown are representative from 2 biological replicates.

E. coli. Folinic acid is also known as formyl-tetrahydrofolate monoglutamate, and is given as a clinical folate supplement to patients taking MTX, to overcome the cytotoxicity and side effects that MTX may have (Chabner et al. 1985; Whittle & Hughes 2004). Folic acid, an oxidised monoglutamate folate commonly used as a supplement can also be given to people taking MTX. To investigate whether folic acid supplementation would rescue the growth phenotype of the *gcp-2.1* mutant, worms were maintained on SMX-treated *E. coli*, that had also been supplemented with folic or folinic acid. Data show that whilst 10 μM folinic acid fully rescued the growth defect in concurrence with previous observations, a concentration of 250 μM folic acid was required for a comparable rescue of growth – research carried out by Claire Maynard (Figure 41). As described in chapter 3, folic acid is a chemically synthesised supplement, at this high concentration, it is possible that there are small amounts of the para-aminobenzoyl with a glutamate (PAB-glu) breakdown product, that is taken up by *E. coli* and fed into the folate cycle (Carter et al. 2007). Thus, the growth rescue observed in the *gcp-2.1* mutant *C. elegans* at 250 μM folic acid supplementation may be due to the PAB-glu restoring the folate status of the *E. coli*, not the folic acid itself. This data suggests that folinic acid is more potent than folic acid, and can restore animal folate status back to levels were healthy growth is possible, at relatively low concentrations. This is expected as *folt-1, 2* and *3* are reduced folate carriers. Folate quantification by LC-MS may provide more information.

Since folinic acid is able to rescue the growth defect in *gcp-2.1* worms, it shows that it can be used as a folate supplement to restore folate levels. This makes us question whether folinic acid supplementation, to wild type worms maintained on SMX treated *E. coli* would rescue the extended lifespan induced by SMX supplementation.

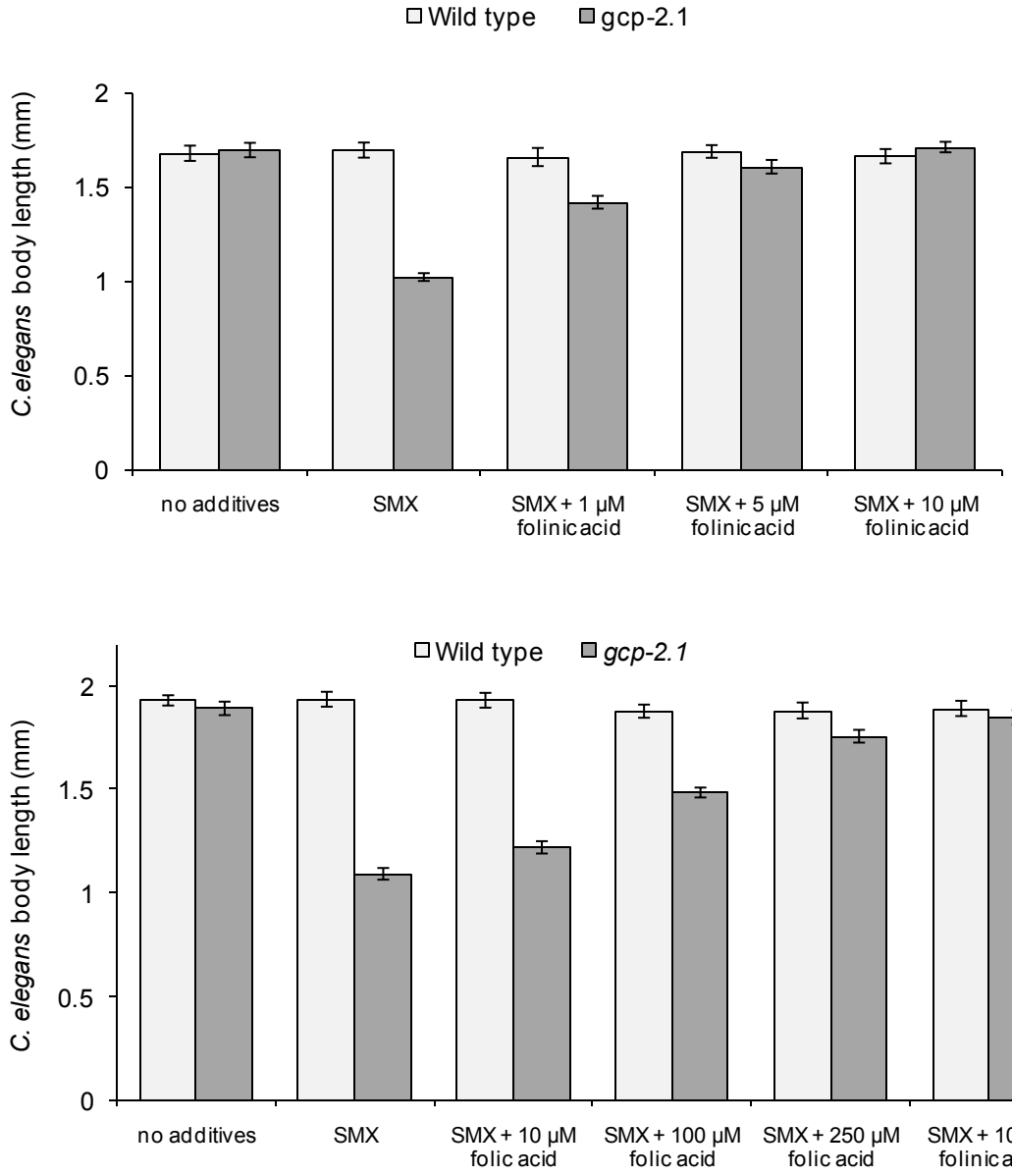


Figure 41: The growth defect observed in *gcp-2.1* mutant *C. elegans* maintained on SMX treated OP50 *E. coli*, can be rescued with folate supplementation. Data obtained and analyzed by Claire Maynard. High purity agar and peptone media used.

Lifespan analysis was performed on *C. elegans* maintained on 128 μg/ml SMX, and SMX treated *E. coli* with either 5 μM or 10 μM folinic acid supplementation. Data show that 10 μM folinic acid, the concentration shown to rescue growth defects due to decreased folate levels, does not significantly affect the lifespan extension caused by SMX supplementation (Figure 42). Surprisingly, 5 μM folinic acid supplementation appears to further increase the lifespan extension observed with SMX treatment (SMX mean lifespan = 19.31 days, 5 μM folinic acid + SMX mean lifespan = 21.60 days, $P<0.0001$) (Figure 42).

5.3 Discussion

Here I have used a chemical intervention, in the form of MTX in attempt to inhibit folate synthesis in *C. elegans,* and characterised a *C. elegans* mutant, that is thought to inhibit folate uptake. Results of this study show that neither inhibition of the animal folate cycle, nor folate uptake in the animal result in increased longevity. We must therefore assume that the folate dependent action resulting in an increase in *C. elegans* lifespan is due to altered folate status in the *E. coli*, which the worms are fed on. As neither folinic acid nor folic acid can be taken up by *E. coli*, any effects of supplementation are purely due to restoring folate levels in *C. elegans*. I have presented data suggesting that folate supplementation, may not affect the lifespan enhancing property of *E. coli* with excess folate removed. Data from 5 μM folinic acid show that folate supplementation might further increase the lifespan, however to be certain that this effect is real; the experiment would need to be repeated. Perhaps 5 μM folinic acid is an adequate concentration to partially restore folate levels in the worm, but not completely. The growth data of *gcp-2.1* indicates that 5 μM folinic acid supplementation is not sufficient to rescue growth fully. Maybe an intermediate folate

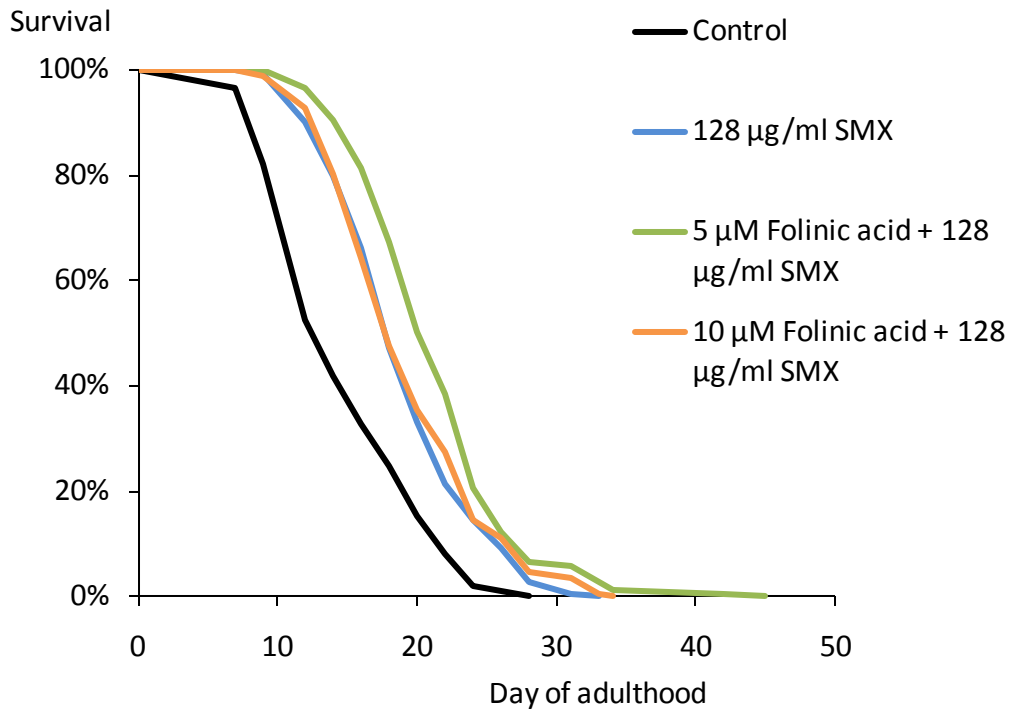


Figure 42: Concentrations of folinic acid that rescue the *gcp-2.1* mutant growth defect on SMX treated OP50, do not rescue the lifespan increase caused by SMX. Lifespan analysis performed on peptone high purity agar. *C. elegans* strain used SS104, *E. coli* strain used OP50. Control n = 138 mean lifespan = 15.00 days, 128 µg/ml SMX n = 153 mean lifespan = 19.31 days (P<0.0001), 5 µM folinic acid + 128 µg/ml SMX n = 171 mean lifespan = 21.60 days (P<0.0001), 10 µM folinic acid + 128 µg/ml SMX n = 145 mean lifespan = 19.66 days (P<0.0001).

status between the low levels induced by SMX, and the excessive levels under normal bacterial conditions brings health benefits directly to the animal. Data presented here show that folinic acid is a much more potent supplement than the perhaps more commonly used folate supplement folic acid, as in this system folinic acid is more effective at restoring animal folate levels. Folate measurement by LC-MS would provide confirmation of these findings. The human orthologue of *folt*-1(RFC) absorbs mono-glutamated and di-glutamated folate species from the gut. RFC has a much higher affinity for folinic acid than folic acid. In humans there are other folate uptake systems at work, which in contrast to RFC have a higher affinity for folic acid than folinic acid. In *C. elegans* whilst there are two putative folate transporters; evidence suggests that *folt-1* is the main transporter. If so, then this could explain why folinic acid supplementation shows rescue at much lower concentrations than folic acid, because the *folt-1* folate transporter preferentially transports folinic acid.

CHAPTER 6

INVESTIGATING THE RELATIONSHIP BETWEEN SMX AND KANAMYCIN

6.1 Introduction

C. elegans in the laboratory are usually fed on established lawns of *E. coli*. Studies have shown that the *E. coli* must be live and metabolically active to provide the required nutrition for healthy growth and development of *C. elegans* (Lenaerts et al. 2008). Previous research has shown that when *C. elegans* are fed dead bacteria, which have been killed by antibiotics or UV radiation, the worms exhibit longer lifespan (Gems & Riddle 2000; Garigan et al. 2002). There is also evidence to show that the variation in *C. elegans* lifespan increases in liquid, bacteria free, chemically defined medium when compared to the lifespan of animals raised on bacterial lawns on plates (Szewczyk et al. 2006; Pincus & Slack 2010). These studies imply that whilst *C. elegans* have an essential requirement for live bacteria in order for healthy development, the bacteria have a life shortening effect on the worm.

6.1.2 Bacterial accumulation and ageing

It has been shown that *E. coli* accumulate in the intestinal lumen of ageing adult *C. elegans* (Garigan et al. 2002; McGee et al. 2011). In this field, there is a common assumption that the bacterial accumulation observed in ageing *C. elegans* is a major cause of death. There have been research supporting this model, showing a negative correlation between bacterial accumulation level and *C. elegans* longevity (Baeriswyl et al. 2010; Portal-Celhay et al. 2012; Gomez et al. 2012). However, it is worth noting that the increase in bacterial accumulation as worms age, may be due to the ageing of

the intestine which loses integrity as *C. elegans* age, and thus may become more susceptible to bacterial accumulation (Herndon et al. 2002; Pincus & Slack 2010; McGee et al. 2011). As worms progress through adulthood muscle integrity has been shown to decrease, leading to a reduction in motility. Alongside this sarcopenia, it has been shown that the pharyngeal contraction rate decreases, which could also lead to older *C. elegans* being more likely to accumulate bacteria in their intestine (Chow et al. 2006). Therefore bacterial accumulation may be a biomarker rather than a cause of ageing.

It has been suggested that the antibiotic kanamycin, increases *C. elegans* lifespan by preventing bacterial accumulation in the intestine (Garigan et al. 2002). Kanamycin does prevent bacterial proliferation by inhibiting translation (Garigan et al. 2002), however to the best of our knowledge it has never been proven that specifically preventing bacterial accumulation results in an increased *C. elegans* lifespan.

In the previous chapter, data show that alterations in microbial folate synthesis, rather than *C. elegans* folate status, cause an increased animal lifespan. It is important to note that whilst sulfamethoxazole (SMX) does not slow bacterial growth in this system (Figure 31, 32) literature shows that it is an antibiotic. It is possible that antibiotic characteristics of the drug may play a role in the robust increase in longevity observed in *C. elegans* maintained on SMX-treated *E. coli*.

6.1.3 Aims and Objectives

In this chapter I aim to look further into the mechanism via which SMX works to increase animal lifespan. By comparing the SMX-induced increase with a widely

accepted mechanism of lifespan extension, such as maintaining *C. elegans* on kanamycin treated *E. coli*. Secondly I aim to explore whether SMX influences bacterial accumulation in the intestinal lumen of *C. elegans*.

6.2 Results

6.2.1 Kanamycin and SMX have similar effect on lifespan

In attempt to provide more information about SMX and the way in which it extends *C. elegans* lifespan, we wanted to compare the increase in lifespan observed with SMX treatment to the increased longevity seen when kanamycin treatment is applied to an established lawn of *E. coli*. If SMX supplementation were to further extend the increased lifespan seen with kanamycin treatment alone, it would suggest that both drugs are working via different pathways. However if when both drugs are combined, the increase in animal lifespan is not additive, it implies that both drugs may be working via a common mechanism or pathway. Pooled results from four experiments show that the increase in lifespan caused by kanamycin treatment is indistinguishable from the lifespan extension seen with SMX treated OP50 *E. coli*. When both drugs were combined there was a slight increase of approximately 1 day in mean lifespan than with either drug alone (Figure 43). Statistical analysis shows that the difference between either drug alone and the combination of both SMX treatment and kanamycin treatment is significant (P=0.0008). It is worth noting that statistical analysis was performed on data pooled from 4 experiments showing the same trend in data resulting in large sample sizes, therefore any perturbations would have a large effect on the statistics used. The lifespan curve (Figure 43) shows that the increase in lifespan when both drugs are used is minimal as the beginning and end of lifespan is very similar. After careful consideration and reviewing the data we assume that there

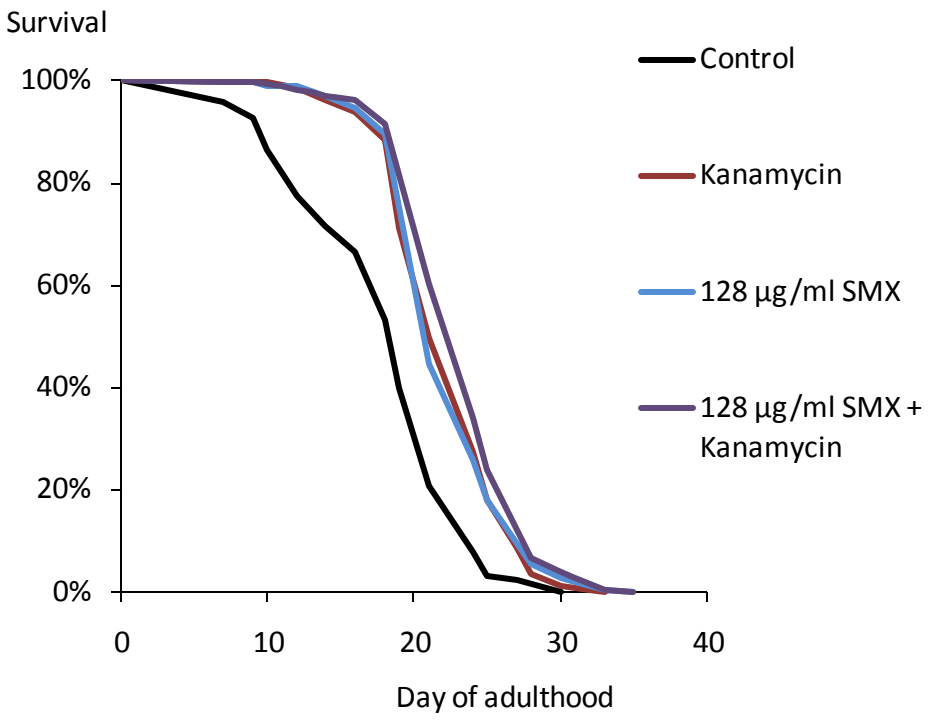


Figure 43: SMX and kanamycin exhibit an identical effect on *C. elegans* lifespan. Lifespan analysis performed on peptone high purity agar. Kanamycin treatment was 80 µl of 10mM Kanamycin added to the bacterial lawn 2 days after seeding. *C. elegans* strains used SS104, *E. coli* strains used OP50. Control (OP50) n = 423 mean lifespan = 17.78 days, Kanamycin n = 466 mean lifespan = 22.28 days ($P<0.0001$), 128 µg/ml SMX n = 469 mean lifespan = 22.29 days ($P<0.0001$), 128 µg/ml SMX + Kanamycin n = 481 mean lifespan = 23.21 days ($P<0.0001$). Data shown are representative from 4 biological replicates.

is no additive effect of both drugs. This data is surprising as the non additive effect on *C. elegans* lifespan suggests that both drugs are working via a common mechanism. Consistent with this hypothesis, the lifespan extension of either SMX or kanamycin alone are almost identical. The question arises, how SMX and kanamycin can be working through the same mechanism to extend lifespan, when kanamycin is known to prevent proliferation of bacteria, whilst our data has shown that SMX has minimal effect on *E. coli* proliferation on plates (Figure 32) and no effect in liquid culture (Figure 31). Perhaps SMX inhibits a particular life-shortening activity of *E. coli*, if so then by blocking proliferation kanamycin might also inhibit the life-shortening activity.

6.2.2. Does SMX increase lifespan by preventing *E. coli* accumulation?

It has been suggested that kanamycin increases the lifespan of *C. elegans* by preventing bacterial accumulation in the intestinal lumen. Bacterial accumulation is thought to be a major cause of animal death via catastrophic bacterial infections as a result of tight packing in the accumulated area (Garigan et al. 2002). Given the identical effect in lifespan, it may be that SMX treatment extends *C. elegans* lifespan via a similar mode of action, by reducing bacterial accumulation within the lumen of the worm. To test this hypothesis, bacterial accumulation assays were performed using green fluorescent protein (GFP)-tagged *E. coli* which allowed us to visualise areas of accumulation (see section 2.18).

6.2.3 SMX increases GFP expression of OP50-GFP

When preparing for the bacterial accumulation assays, we observed a striking difference in the levels of GFP expression between *E. coli* that had been treated with SMX and control *E. coli* (Figure 44). To quantify this observation, *E. coli* lawns were grown on SMX supplemented plates, and after 2 days when the lawns had established the intensity of GFP expression was measured by taking images of the lawns and measuring the intensity (section 2.17). Data show that even at low concentrations such as 1 μg/ml SMX, the brightness of GFP observed was substantially higher than observed in control *E. coli* with no SMX supplementation. There was a dose dependent increase in GFP intensity. Whilst analysing these plates, we observed that as well as an increase in GFP intensity, the edges of the bacterial lawns appeared to become smoother with SMX supplementation (Figure 44b). This change in *E. coli* lawn morphology perhaps indicates that the SMX is having a subtle effect on the bacteria shape or adhesion, SMX-treatment does cause a change in *E. coli* cell morphology.

We wanted to determine whether this increase in GFP intensity was a characteristic of sulphonamides in general, perhaps sulphonamides cause an increase in transcriptional response of the *E. coli*. In order to test this we performed the same bacterial lawn assay using GFP-tagged OP50 treated with another sulfonamide, sulfaguanidine (SFG). When compared to SMX, SFG showed no significant increase in GFP intensity when compared to control, even at the highest dose (Figure 45). Whilst SFG is only one of the many sulfonamides, this result does suggest that not all sulfonamides have the same effect on transcription, and therefore the increase in GFP intensity observed with SMX supplementation is not a general characteristic of all sulfonamides.

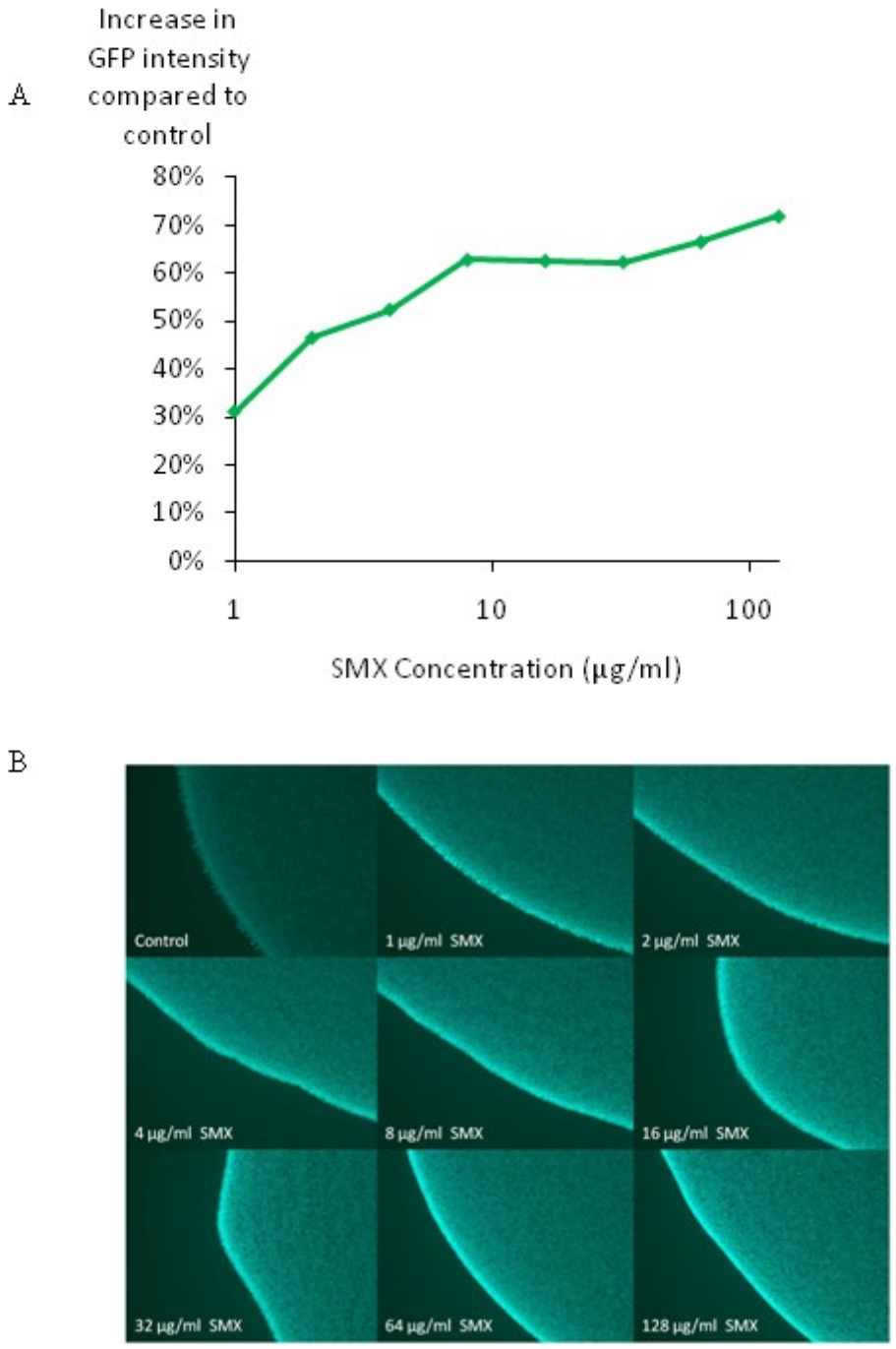


Figure 44: SMX treatment induces a dose dependent increase in OP50 E. *coli* GFP intensity. High purity agar and peptone media used A) Graph showing percentage increase in GFP intensity as compared to the control (no SMX supplementation). B) Images of GFP bacterial lawn excluding the edges were used to quantify GFP intensity. Data shown are representative from 3 biological replicates.

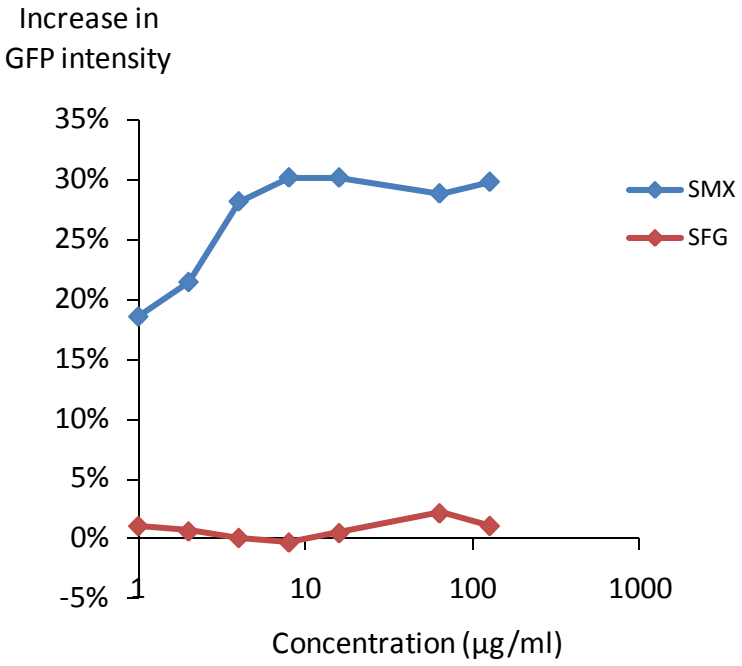


Figure 45: Sulfaguanadine does not induce an increase in OP50 *E. coli* GFP intensity. High purity agar and peptone media used. Concentrations used 1, 2, 4,8,16, 64 and 128 µg/ml of each sulfonamide respectively. Percentage increase of GFP calculated by comparing mean GFP intensity treated OP50 with base level GFP intensity of untreated OP50 *E. coli*.

6.2.4 Assessing bacterial accumulation on our system

Bacterial accumulation assays were performed, scoring adult *C. elegans* as either no accumulation, partial accumulation or full accumulation (Figure 46). These classes were used, as heterogeneity in levels of accumulation was observed and these classes allowed the least subjective scoring of individuals. In these assays only *E. coli* accumulation in the intestinal lumen of the worms was recorded, and bright spots in the pharynx area were not included. Scoring of animals was performed using a long pass Leica GFP2 filter that allows all long wavelengths through, allowing a clear distinction between *E. coli* GFP expression (bright green), and autofluorescence (which appeared more yellow in colour) (Figure 47). Worms were observed from day 1 of adulthood for accumulation during these assays. Worms began to accumulate *E. coli* from day 5 of adulthood. Whilst our finding suggest that SMX does not appear to prevent bacterial accumulation as kanamycin is reported to, SMX does seem to delay the onset of bacterial accumulation (Figure 48). Comparing replicates of the assay shows that accumulation patterns are similar between live worms in different experiments.

If bacterial accumulation was a major cause of death, it may be expected that there would be an increase in accumulation with time, with all worms showing high levels of accumulation in the later stages of adulthood, our data shows that the number of worms with full accumulation appears to decrease with age. Approximately 40% of recently dead worms show no signs of accumulation (Figure 49).

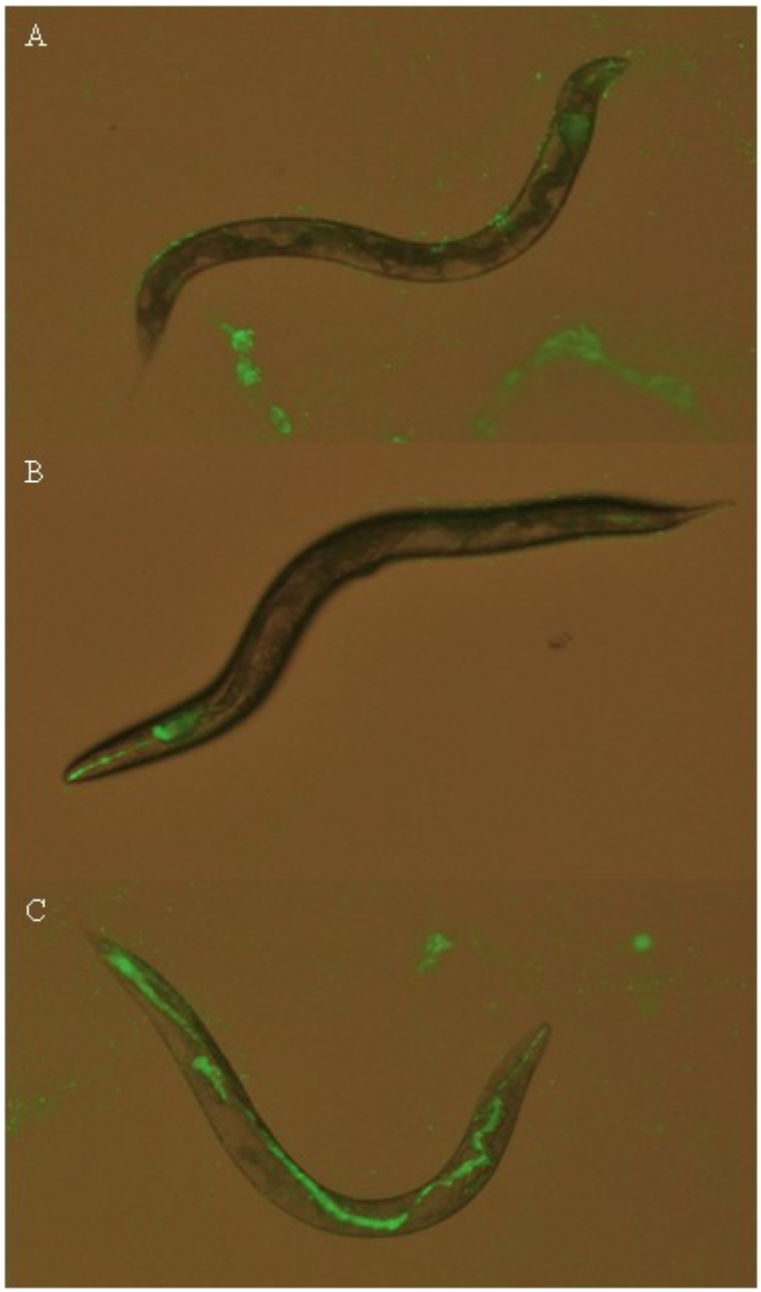


Figure 46: Scoring of *C. elegans* intestinal GFP bacterial accumulation. High purity agar and peptone media used. *C. elegans* strain used SS104; *E. coli* strain used OP50-GFP. Representative images of the 3 classes of accumulation scored in this study A) No accumulation, B) Partial accumulation, C) Full accumulation. Images taken on day 6 of adulthood.

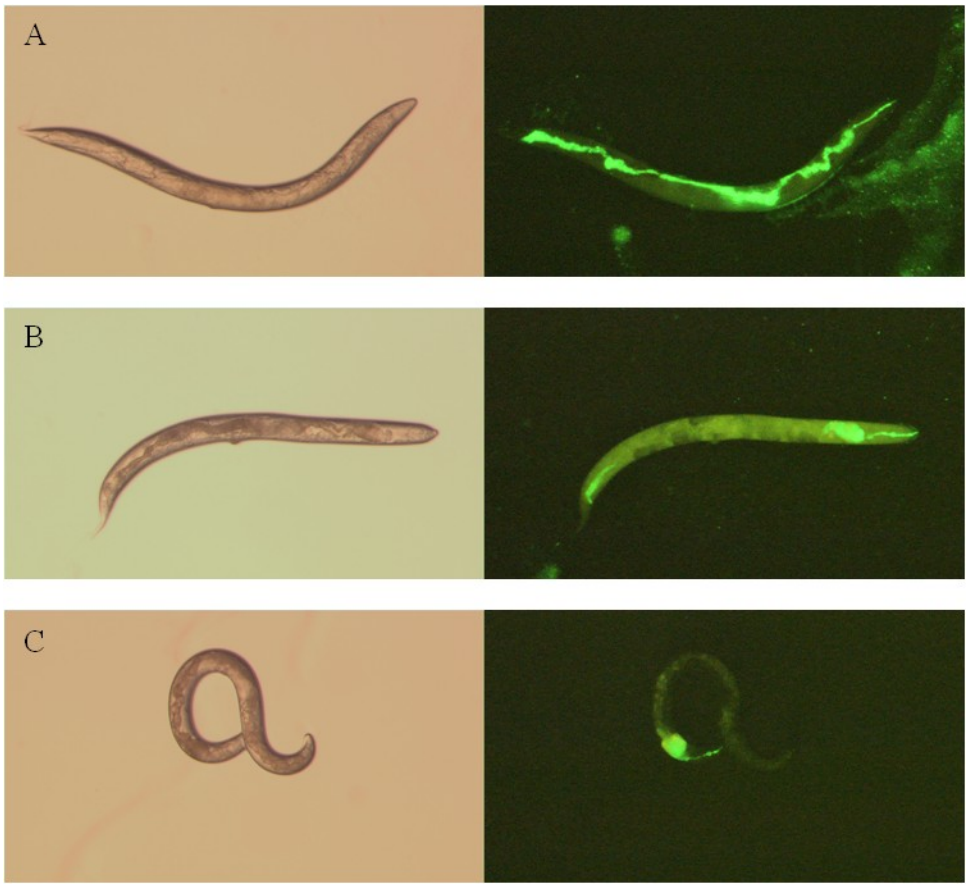


Figure 47: Use of a wide pass filter enables distinction between GFP bacterial fluorescence and autofluorescence. Representative images depicting different autofluorescence. High purity agar and peptone media used. A) No autofluorescence, green observed is GFP bacterial fluorescence, B) Some GFP bacterial fluorescence observed (green), most of the worm shows autofluorescence (yellow), C) Two spots of yellow autofluorescence in the pharynx region, intermittent with green bacterial fluorescence. Images taken on day 6 of adulthood.

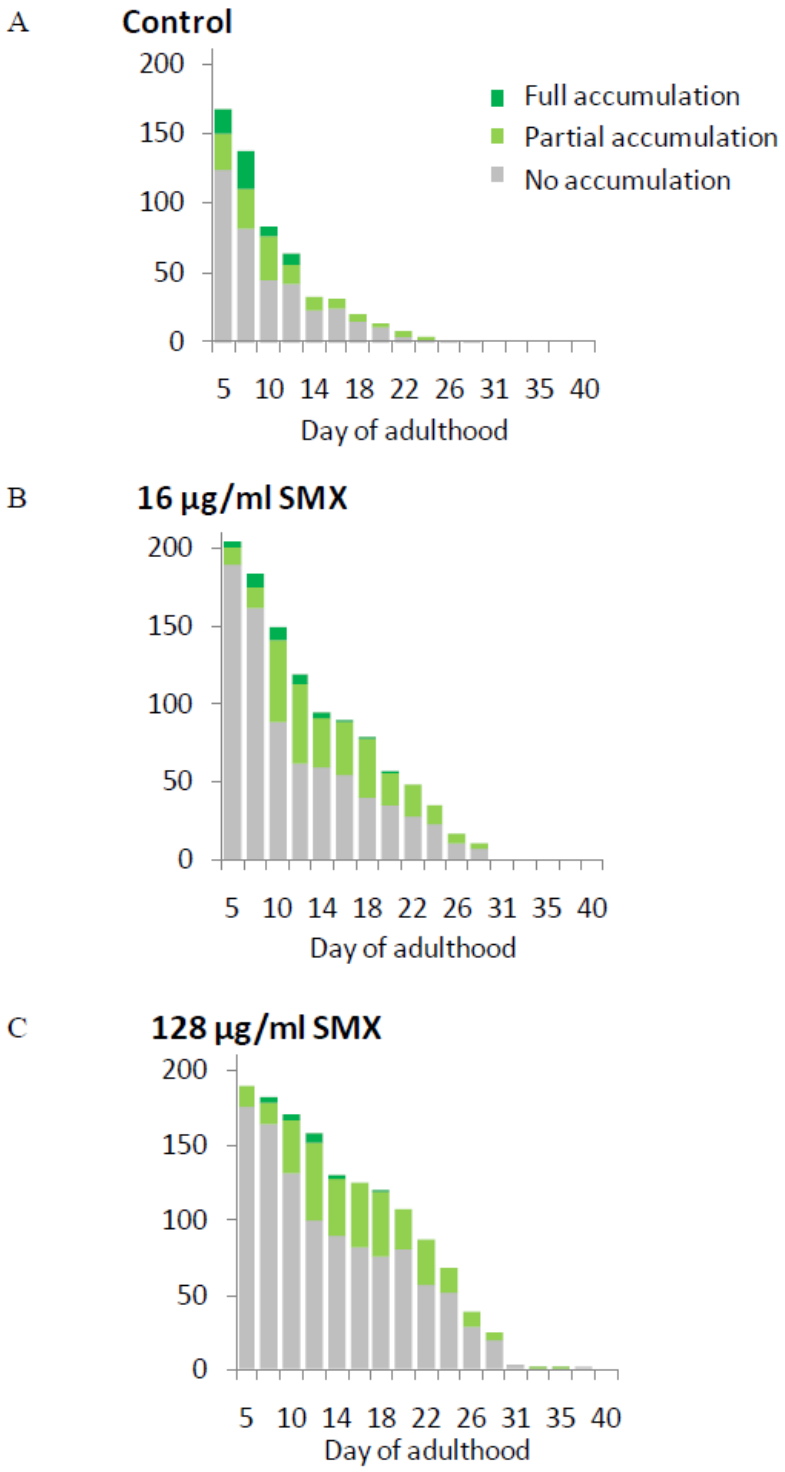


Figure 48: Levels of GFP bacterial accumulation in the intestinal lumen of live worms throughout adult lifespan. High purity agar and peptone media used. *C. elegans* strain SS104, *E. coli* strain OP50-GFP. Y axis represents number of worms. Representative data from 3 biological replicates are shown.

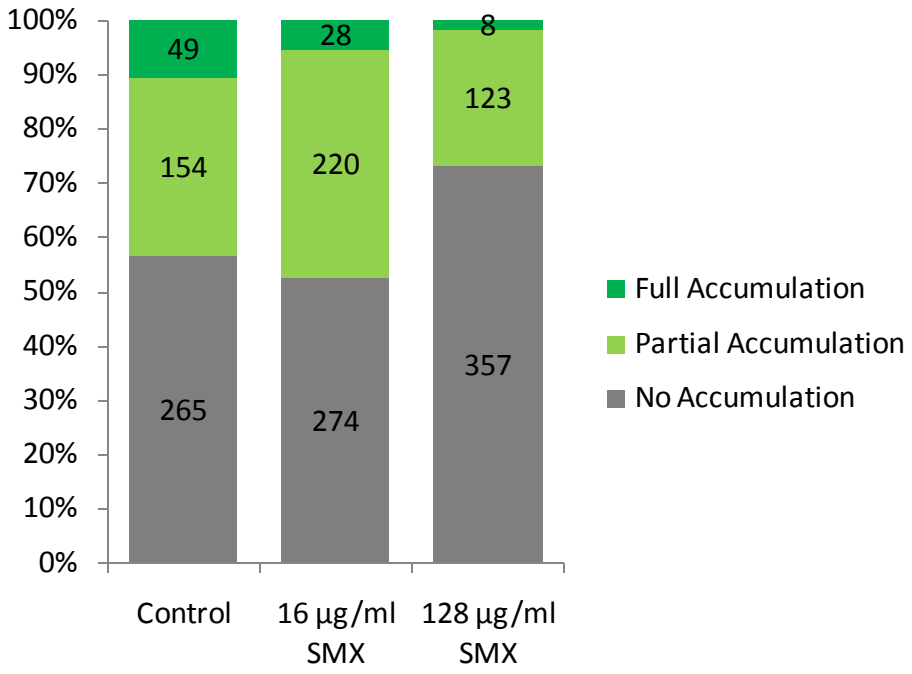


Figure 49: Levels of GFP bacterial accumulation in the intestinal lumen of recently dead worms throughout lifespan experiment. High purity agar and peptone media used. *C. elegans* strain SS104, *E. coli* strain OP50-GFP. Representative data from 3 biological replicates are shown.

6.2.5 Bacterial accumulation in this system does not appear to be an early predictor of ageing

Lifespan analysis was performed to further investigate whether bacterial accumulation was a predictor of lifespan. *C. elegans* were grown and then sorted on day 6 of adulthood into groups based on the level of bacterial accumulation. For this particular lifespan assay, *C. elegans* with either very low levels of bacterial accumulation or very high levels of bacterial accumulation were used. For animals with low levels of accumulation, typically accumulation was observed in a small part of the intestine, either at the tail end of the animal or at the start of the lumen near the pharynx. Animals with high levels of accumulation typically showed full accumulation. These low accumulation and high accumulation grouped *C. elegans* were then maintained on plates with or without SMX supplementation. Results show that worms with low levels of accumulation did not live significantly longer than worms with higher levels of accumulation, consolidating our hypothesis that bacterial accumulation may not be a major cause of death in worms. This finding suggests that bacterial accumulation may not be an early life predictor of ageing. This data also shows that SMX extends lifespan regardless of bacterial accumulation level, suggesting that SMX does not work by reducing bacterial accumulation (Figure 50). Unfortunately the numbers of animals used in this analysis were slightly lower than usual, particularly in the high accumulation groups, as there were very few worms in the populations grown showing very high levels of accumulation at the point of lifespan set up – day 6 of adulthood. To be more confident in this result, this experiment needs to be repeated.

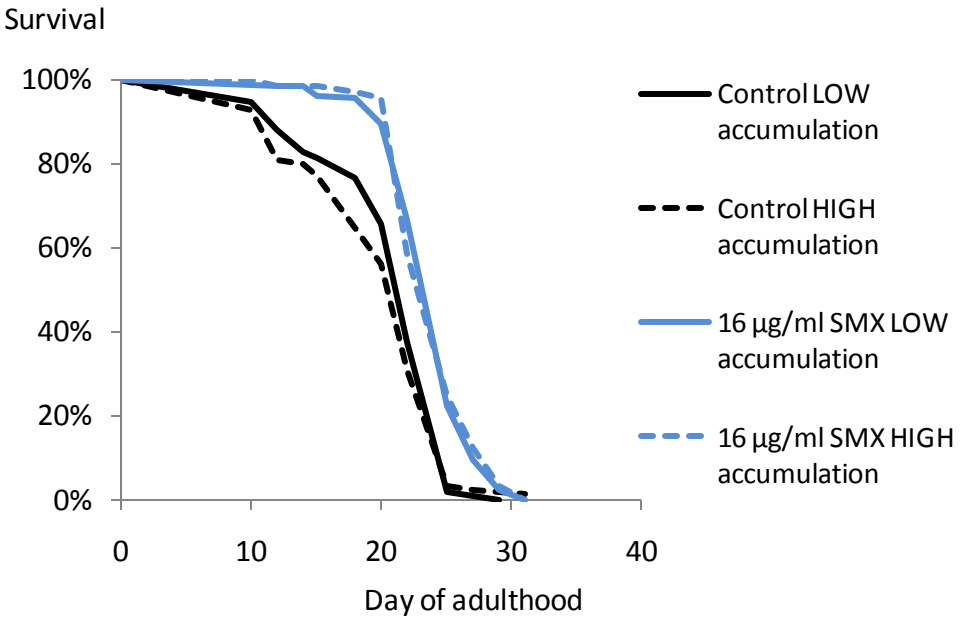


Figure 50: Level of bacterial accumulation does not affect lifespan. Lifespan analysis performed on peptone high purity agar. *C. elegans* strain used SS104; *E. coli* strain used OP50-GFP. Control low accumulation n = 66 mean lifespan = 20.96 days, Control high accumulation n = 98 mean lifespan = 20.06 days (n.s), 16 µg/ml SMX low accumulation n = 130 mean lifespan = 24.21 days (P<0.0001), 16 µg/ml SMX high accumulation n = 65 mean lifespan = 24.31 days (P<0.0001).

6.3 Discussion

Here we have shown that SMX and kanamycin treatment appear to have an identical effect on *C. elegans* lifespan. However when both drugs are combined, there is no further increase in lifespan. *E. coli* accumulation assays with SMX supplementation showed that many worms die without any apparent accumulation. Whilst SMX did not prevent bacterial accumulation, it did appear to prolong the onset of bacterial accumulation in adult worms leading to the hypothesis that rather than being a cause of death, bacterial accumulation may be a bio marker of ageing.

Whilst performing bacterial accumulation assays the observation was made that GFP-tagged OP50 grown on plates with SMX supplementation appeared brighter than control OP50. After reviewing literature we made the assumption that this was due to an increase in transcriptional response of the *E. coli* to SMX, and tested SFG another sulfonamide to see whether it had a similar effect. Although SFG did not increase GFP intensity, lifespan analysis of was not performed on *C. elegans* maintained on SFG treated *E. coli*. To provide more information lifespan analysis should be performed on SFG supplemented *E. coli*, folate levels of SFG-treated *E. coli* should be measured, and other sulfonamides could also be investigated.

During our bacterial accumulation assays a wide pass filter was used which allows distinction between green GFP expression from the accumulated *E. coli*, and yellow autofluorescence. In previous studies looking at bacterial accumulation, autofluorescence has not been overly mentioned. In our research we found that many worms showed high levels of autofluorescence shortly before death and for a short

time period afterwards. This means that if a narrow pass filter, not able to easily distinguish between autofluorescence and GFP-expression was used, this high level of autofluorescence may be mistaken for a bacterial infection, with GFP-expressing bacteria having infected the entire worm body.

Bacterial accumulation assays were performed using GFP tagged *E. coli*. As mentioned SMX treated *E. coli* are brighter than control OP50. Whilst this means that the increase in brightness simplifies the scoring of accumulation in *C. elegans* maintained on SMX treated *E. coli*, it means that because of this increase there may be a slight bias in our results. In theory the same small number of accumulated *E. coli* would be easier to see in SMX treated condition compared to the control. However it remains to be seen whether results would be affected. The accumulation assays were performed under optimised lighting conditions, which meant that the GFP-tagged *E. coli* were highly visible whether they were treated with SMX or not. Colony forming units (CFU) assays could be performed to determine that the observed bacteria correlate with CFU counts. Studies that have performed CFU counts report that they use 5 or 10 worms. In this assay, it would be useful to be able to perform CFU counts on single worms.

Lifespan analysis of worms with either low levels of bacterial accumulation, or high levels of bacterial accumulation on day 6 of adulthood was performed both with and without SMX supplementation. Whilst results showed that the worms with high accumulation did not live significantly longer than those with low levels of accumulation, it is worth noting that worms used for this particular lifespan were classed into bacterial accumulation categories (either high or low) on day 6 of

adulthood when the lifespan experiment as initially set up. After this the bacterial accumulation level was not monitored. As bacterial accumulation can vary from day to day especially in the early stages of adulthood, which is when the worms were classified by accumulation level, perhaps instead of making the conclusion that bacterial accumulation level does not correlate with lifespan, the real conclusion of this particular experiment is that accumulation level at day 6 does not have an impact on final longevity. Unfortunately the numbers of animals in this analysis were slightly lower than usually used in the high accumulation groups, as there were very few of the worms in the populations grown showing very high levels of accumulation at the point of lifespan set up. Therefore to be more confident in this result it would have to be repeated. However, in spite of these doubts, given other data from the bacterial accumulation assays we can still conclude that severe bacterial accumulation may not be a major cause of death in this system, rather a marker of ageing like motility.

CHAPTER 7

GENERAL DISCUSSION

7.1 Overview of results

The initial finding leading to this project was the chance discovery and the characterisation of the *E. coli aroD* mutant. When *C. elegans* were maintained on the *aroD* mutant they show extended lifespan. The *aroD* gene codes for the enzyme 3-dehydroquinate dehydrotase, essential in the shikimic acid pathway, the pathway that produces chorismate the precursor to all aromatic compounds including folates. Supplementation with para-aminobenzoic acid (PABA) completely rescues the lifespan extension, suggesting that the *aroD* mutant *E. coli* causes its effect on lifespan in a folate specific manner. To further investigate this, varying concentrations of exogenous folates were supplemented to the media. Folic acid supplementation was shown to partially rescue the extended *C. elegans* lifespan with lower concentrations of exogenous folic acid. With high concentrations of folic acid supplementation or folinic acid at supplementation, lifespan was not significantly different from the control, confirming that the lifespan increase is caused by a folate specific mechanism.

The lifespan effect caused by the *aroD* mutant was found to be sensitive to perturbations in media. The enzyme encoded by *aroD*, is upstream in the shikimic acid from folates, and thus it was possibly that perhaps the mutation in *aroD* might be affecting other processes as well as folate synthesis. Due to these drawbacks of the novel mutant, I aimed to find a pharmalogical intervention that more specifically targeted folate synthesis, and to determine whether this intervention would cause a similar increase in *C. elegans* lifespan than that observed with *aroD* mutant *E. coli*. I

focused on the sulfonamide sulfamethoxazole (SMX), which exhibits a dose dependent increase in *C. elegans* lifespan maintained on SMX-treated OP50, without being sensitive to media type. The increase in lifespan observed in *C. elegans* with SMX supplementation can also be fully rescued by the addition of PABA, implying that the lifespan effect is folate dependent.

To investigate any changes in metabolism with regards to the *aroD E. coli* mutation and SMX treatment, I used liquid chromatography coupled to mass spectrometry (LC-MS) to measure and quantify folate levels in both *E. coli* and worms. Folate analysis showed that formyl tetreahydrofolate with 3 glutamates (formylTHFGlu$_3$) was the most abundant folate species observed in *E. coli*. The *aroD* mutant *E. coli* contained significantly lower levels of formylTHFGlu$_3$ than the wild type HT115(DE3) strain. In *C. elegans* the most abundant folate observed using our method of extraction of analysis was 5-methyl tetrahydrofolate with 5 glutamtes (5-methylTHFGlu$_5$). Worms maintained on *aroD* mutant *E. coli* also showed significantly lower levels of 5-methylTHFGlu$_5$. Folate measurements using SMX treated OP50 showed similar patterns to the *aroD* mutant, significantly lower levels of the most abundant folate species in both worms and *E. coli*. Levels of formylTHFGlu$_3$ in *E. coli* treated with high dose SMX (128 μg/ml) were beyond the limit of detection. Folate analysis of *E. coli* and worms grown on a range of SMX concentrations showed that SMX supplementation above 1 μg/ml resulted in the levels of the most abundant folate levels in both *E. coli* and *C. elegans* being decreased to a level below the limit of detection.

With such drastic decreases in the levels of folate, it raises the question whether this change in folate status has any deleterious side effects on bacterial growth, or *C. elegans* growth and reproduction. SMX supplementation causes a minimal effect on bacterial lawn density, but causes no proliferation defects. Furthermore, *C. elegans* maintained on SMX treated OP50 show no significant change in development time, reproductive cycle or total brood size. To examine whether SMX exhibited any negative side effects later in *C. elegans* life, I used at worm motility as a marker of ageing. *C. elegans* maintained on SMX treated *E. coli*, show higher levels of motility later into adulthood. This result implies that SMX prolongs ageing in worms.

Thus far, data had shown that a change in folate status can cause extended longevity in *C. elegans*. The exact mechanism however remained unclear. The drug methotrexate (MTX) was employed as an intervention that would specifically target and inhibit the *C. elegans* folate cycle. MTX did not cause a significant increase in worm lifespan, implying that inhibition of the *C. elegans* folate cycle alone does not extend longevity.

To determine whether impeded folate uptake in the animal would have an effect on lifespan we used the *gcp-2.1* worm mutant. The *gcp-2.1* gene codes for the enzyme GCPII which cleaves glutamate molecules from polyglutamated folates. The removal of glutamate molecules is essential, as it has been reported that folate transporters show higher affinity for monoglutamated folate species. Results showed that *gcp-2.1* mutant worms did not exhibit any significant increase in lifespan, and therefore inhibition of folate uptake in the worm does not extend longevity.

When *gcp-2.1* worms were maintained on SMX treated *E. coli* they showed stunted growth, uncoordinated movement and sterility. This finding provided the first phenotype of SMX supplementation other than extended lifespan, and provides support for the hypothesis that *gcp-2.1* mutant worms have a lack of monoglutamated folate species. The growth deficiency observed in *gcp-2.1* mutant worms was rescuable with folate supplementation. Folinic acid, a monoglutamated form of folate, was a more potent supplement than folic acid, illustrated by its ability to restore healthy growth at low concentrations (5 and 10 μM), which is expected as the folate transporters in *C. elegans* are reported to be reduced folate carriers. As low concentrations of folinic acid supplementation can restore folate status of *gcp-2.1* mutants grown on SMX treated OP50, I investigated whether folinic acid supplementation would rescue the lifespan effect caused by SMX. Folinic acid did not rescue the increased lifespan observed with SMX supplementation.

Finally the SMX induced increase in lifespan was compared to maintaining worms on kanamycin. *E. coli* treatment with either SMX or kanamycin results in an identical increase in *C. elegans*, with no additive effect on longevity when *E. coli* are treated with both SMX and kanamycin. This data suggests that both kanamycin and SMX are working via the same mechanism to increase *C. elegans* lifespan. It is thought that kanamycin causes its effect on lifespan by preventing the accumulation of *E. coli* in the intestinal lumen of ageing *C. elegans*. Bacterial accumulation assays focussed on single worms and followed accumulation throughout adulthood. Data show that approximately 40% of worms die with no visible accumulation, and that SMX does not prevent *E. coli* accumulation, but does prevent the onset of accumulation.

7.2 Other folate related genes in *E. coli* also increase *C. elegans* lifespan

A screen of *E. coli* mutants and their effect on worm lifespan, performed in our lab that two genes involved in folate synthesis, *pabA* and *pabB* increased *C. elegans* lifespan significantly. Both *pabA* and *pabB* are involved in synthesis of PABA. In *E. coli*, PABA is produced from chorismate in two stages. In the first step, the proteins PabA and PabB, coded for by *pabA* and *pabB* respectively, interact to catalyze the transfer of the amino group from glutamine to chorismate (Green et al. 1996; Basset et al. 2004). Interestingly other genes involved in the folate cycle appeared to have no effect on *C. elegans* lifespan, confirming that *E. coli* folate synthesis not the folate cycle is important for lifespan.

7.3 The importance of folate synthesis

All bacteria require folates for growth and synthesis of essential compounds, however not all bacteria have the genes required to synthesize folate. Folate is secreted by many bacteria, including *E. coli* (Iwai et al. 1970; Hoffbrand et al. 1971; Pompei & Cordisco 2007), but other bacteria need to import folate – for example some species of *Lactobacillus* (Rossi et al. 2011). A recent study shows that folate synthesis genes are highly represented in human gut microbes before the age of two years, but after this age show decreased representation, with an increase in the representation of folate salvage genes (Yatsunenko et al. 2012).

Methionine is generated from the folate cycle, and is then converted in s-adenosyl methionine (SAM), the primary methyl donor used in cells. It has been shown previously that methionine restriction causes an increase in lifespan in both rodents

and *Drosophila*. Disruption of *C. elegans* SAM synthase (SAMS-1) has also been shown to increase lifespan (Hansen et al. 2005). Metformin, a drug widely used in the treatment of type 2 diabetes has been reported to extend *C. elegans* lifespan. It has been reported that lifespan increase induced by metformin, is in part due to the effects of the drug on bacterial folate and methionine metabolism (Cabreiro et al. 2013)

7.4 Sulfa-drugs can effect folate synthesis at two different stages

During folate synthesis, dihydropteroate synthase catalyzes the condensation of dihydropterin pyrophosphate and PABA to form dihydropteroate. Sulfonamide drugs, inhibit folate production at this step by competing with PABA, and by acting as a substrate for dihydropteroate synthase (DHPS) itself. The resulting sulfa-dihydropteroate acts as an additional folate inhibitor, competing against dihydrofolate (DHF) (Figure 51) (Patel et al. 2004; Nguyen & Clarke 2012). This finding may help to explain why SMX-treated *E. coli* show a more severe decrease in the level of the most abundant folate than observed in the *aroD* mutant *E. coli*. The *aroD* mutation is broad and directly effects the production of chorismate which produces PABA, which is then converted into folate. Literature suggests that *E. coli* are able to take up PAB-Glu, which can then be used to feed PABA into folate synthesis. Therefore, in *aroD* mutant *E. coli*, traces of PAB-Glu obtained from the media would partly restore folate synthesis. SMX in contrast directly competes with PABA. Therefore PAB-Glu would not restore folate levels as SMX would still compete with PABA. The effect that *aroD* mutant *E. coli*, or SMX treatment have on *C. elegans* lifespan is fully rescued with high concentrations of PABA supplementation. With PABA supplementation, SMX cannot out compete PABA, and this results in less sulfa-dihydropteroate derivative being produced, thus blocking SMX at both action points.

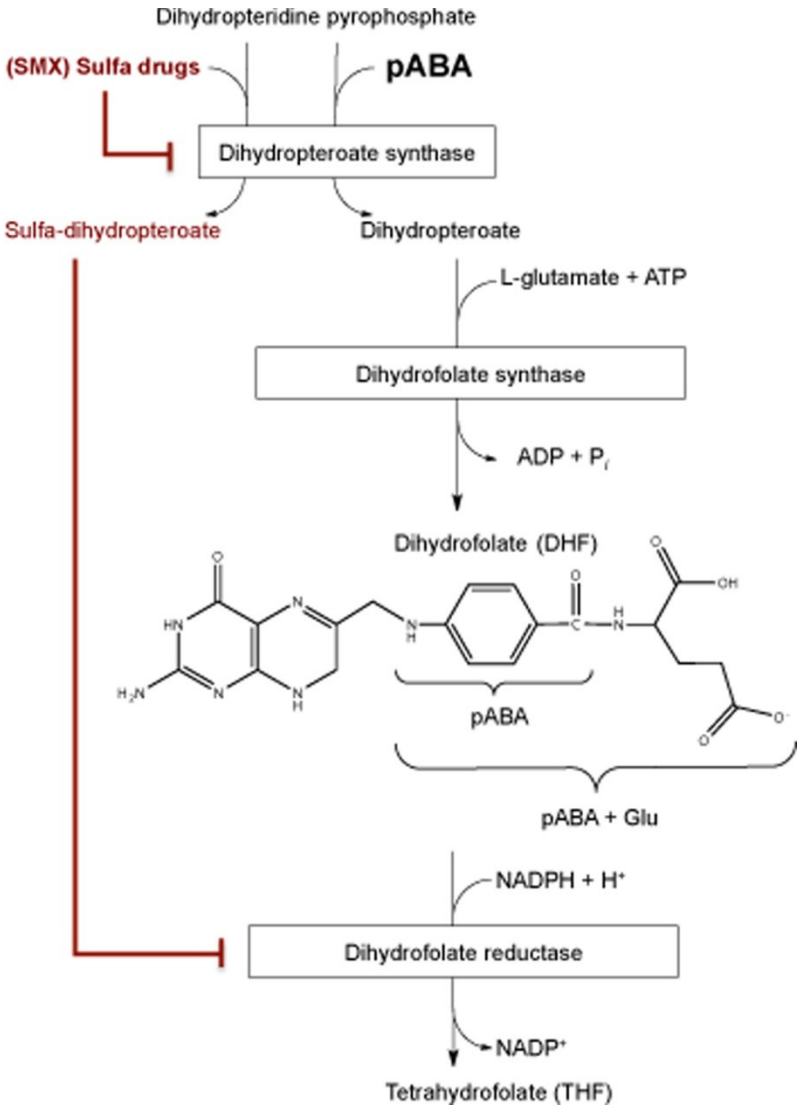


Figure 51: Sulfa-drugs impact folate synthesis at two different points. Image from Nguyen & Clarke, 2012.

7.5 A more sensitive method of folate quantification is required

SMX increases *C. elegans* lifespan in a dose dependent manner, with a concentration of 2 μg/ml, showing a significant and robust increase. Folate analysis shows that treatment of *E. coli* with more than 1 μg/ml SMX decreases the levels of the most abundant folates in both *E. coli* and worms fed on SMX treated *E. coli* to beyond the limit of detection. With such a drastic decrease in folate levels, we would perhaps expect the *E. coli* to exhibit growth defects, however this is not the case. *E. coli* show only a slight decrease in lawn density and no significant change in proliferation, suggesting that folate levels are sufficient for normal growth and cell division. In order to determine the required folate level for growth a far more sensitive method of folate detection would be needed. Perhaps a better folate extraction method could be employed, or the current extraction protocols optimised further. It may also be possible to further adapt the LC-MS method that I have used throughout this research. If the folate detection and quantification was optimised further, then it may provide information on changes in levels of other folate species, rather than only the most abundant folate species. It may also enable detection of monoglutamated folate species, which may be particularly useful for the *gcp-2.1* mutant worm.

7.6 How can SMX and kanamycin work via a common mechanism?

Data presented in chapter 6, indicate that SMX and kanamycin treatment have an identical effect on *C. elegans* lifespan. This is unexpected as SMX treatment does not prevent *E. coli* proliferation, but kanamycin treatment does, so a common mechanism seems unlikely. One possible mechanism could be that in *E. coli*, a folate dependent pathway normally produces an unknown life shortening compound. In this theoretical model, SMX would directly inhibit the folate dependent process and thus inhibit

production of the life shortening compound. In the same manner, by preventing *E. coli* proliferation completely, kanamycin would also inhibit this pathway, and thus the life shortening compound would not be produced. It is important to note also that both drugs are antibiotics, which may lead one to the hypothesis that it is an antibacterial characteristic of these drugs that is causing an increase in animal lifespan. While this may be true as not enough is known about the mechanism via which either drug works, the evidence presented here is substantial and strongly suggests that the mechanism is folate specific (Figure 52).

7.7 Application to mammals

Data presented in this thesis suggest that inhibition of *E. coli* folate synthesis slows animal ageing without any apparent negative effects on the animal or *E. coli* growth. Therefore this type of intervention to target folate synthesis may be possible in the mammalian gut, without causing significant disruption to the gut microbiome. However, different species of bacteria would need to be tested, due to diversity of microbiota present in the gut. Since SMX is an antibiotic it may kill other species of bacteria. Also SMX treatment on a mixture of microbes representative of the gut could be performed, to determine whether the composition of gut microbiota would change. As described in chapter 1, even with varying composition between individuals, it has been shown that prevalence of certain bacterial species can infer a higher risk of disease. If SMX promoted prevalence of such a species it would not be wise to use as an intervention. Research into the effect of sulfonamides on rodents was performed in the 1950's and 1960's. Results show that both rats and mice lived longer when given sulfadiazine (Hackmann 1958). Furthermore, in agreement with my findings, the lifespan increase observed by Hackmann was rescued with PABA supplementation.

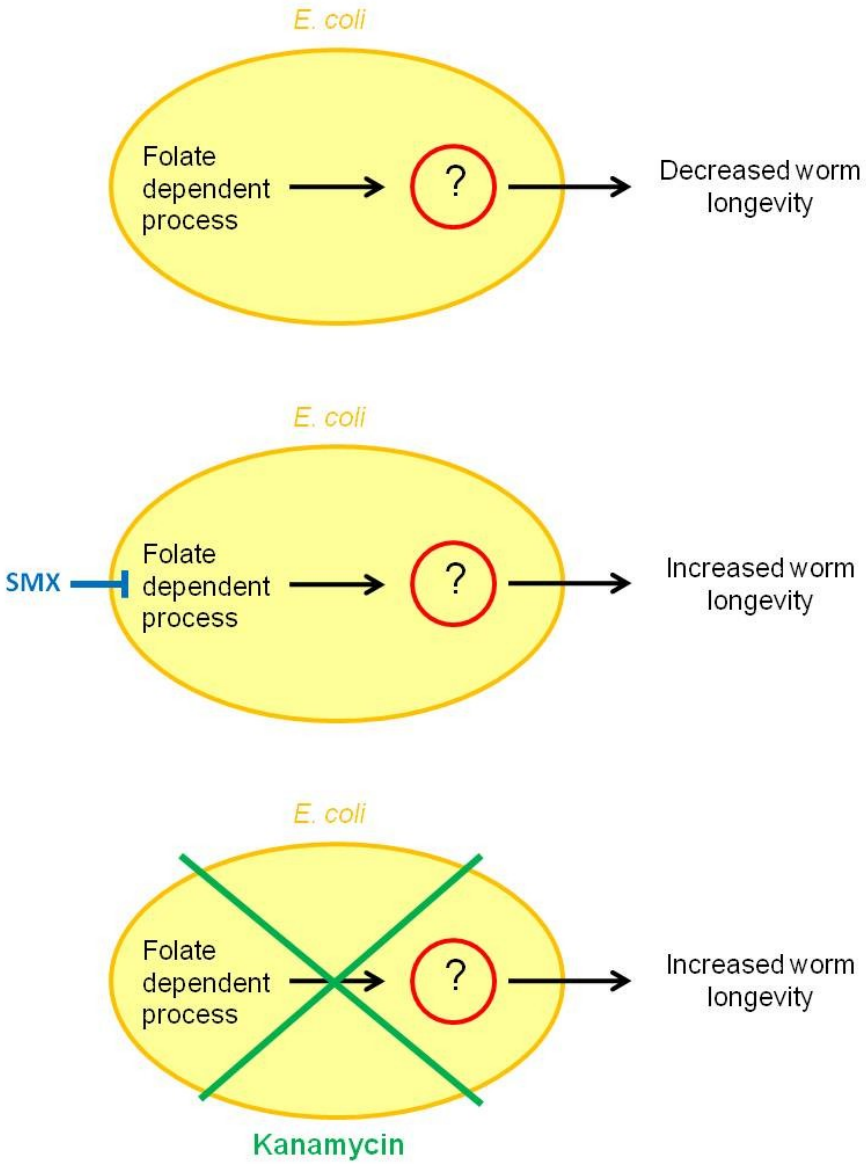


Figure 52: Potential model in which SMX and kanamycin inhibit production of a life shortening compound (denoted by "?") normally produced in *E. coli*

This implies that it is possible to target microbial folate synthesis using sulfonamides in mammals, with a beneficial effect on health. Since data here suggest that inhibition of bacterial folate is the key determinant in lifespan extension, then perhaps other sulfonamide drugs, or alternative drugs that lower folate could be researched into. As some sulfonamides are not absorbed by humans, such a drug would be ideal as it would mean that any side effects to the human host would be minimal, whilst gaining the health benefit of treating gut bacteria and reducing their folate synthesis. As data suggests chapter 5 shows, if SMX were to be used in humans, folinic acid could also be taken to ensure that human folate levels were not deficient as a side effect of gut microbiota, one of our sources of folates, having inhibited folate synthesis.

REFERENCES

Austin, M.U. et al., 2010. Knockout of the folate transporter folt-1 causes germline and somatic defects in C. elegans. *BMC Developmental Biology*, 10(1), p.46. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2874772&tool=pmcentrez&rendertype=abstract.

Baba, T., Ara, T. & Hasegawa, M., 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular systems ....* Available at: http://www.nature.com/msb/journal/v2/n1/synopsis/msb4100050.html [Accessed September 26, 2013].

Baeriswyl, S. et al., 2010. Modulation of aging profiles in isogenic populations of Caenorhabditis elegans by bacteria causing different extrinsic mortality rates. *Biogerontology*, 11(1), pp.53–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19444640 [Accessed September 11, 2013].

Bailey, L.B. & Gregory, J.F., 1999. Folate metabolism and requirements. *The Journal of nutrition*, 129(4), pp.779–82. Available at: http://jn.nutrition.org/content/129/4/779.full [Accessed July 25, 2013].

Balamurugan, K. et al., 2007. Cloning and functional characterization of a folate transporter from the nematode Caenorhabditis elegans. *American journal of physiology. Cell physiology*, 293(2), pp.C670–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17475669 [Accessed July 25, 2013].

Barbieri, M. & Bonafè, M., 2003. Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. *American Journal of* .... Available at: http://ajpendo.physiology.org/content/285/5/E1064?rss=1&ssource=mfc [Accessed May 30, 2014].

Barnett, A.H. et al., 2006. Type 2 diabetes and cardiovascular risk in the UK south Asian community. *Diabetologia*, 49(10), pp.2234–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16847701 [Accessed September 23, 2013].

Barrière, A. & Félix, M., 2005. High Local Genetic Diversity and Low Outcrossing Rate in< i> Caenorhabditis elegans</i> Natural Populations. *Current Biology*. Available at: http://www.sciencedirect.com/science/article/pii/S096098220500655X [Accessed September 24, 2013].

Basset, G.J.C. et al., 2004. Folate synthesis in plants: the p-aminobenzoate branch is initiated by a bifunctional PabA-PabB protein that is targeted to plastids. *Proceedings of the National Academy of Sciences of the United States of America*, 101(6), pp.1496–501. Available at: http://www.pnas.org/content/101/6/1496.short [Accessed September 25, 2013].

Baumeister, R. & Ge, L., 2002. The worm in us–< i> Caenorhabditis elegans</i> as a model of human disease. *Trends in biotechnology*. Available at: http://scholar.google.co.uk/scholar?q=+The+worm+in+us:+Caenorhabditis+elegans+as+a+model+of+human+disease&btnG=&hl=en&as_sdt=0,5#0 [Accessed January 30, 2014].

Beanan, M. & Strome, S., 1992. Characterization of a germ-line proliferation mutation in C. elegans. *Development*. Available at: http://dev.biologists.org/content/116/3/755.short [Accessed September 27, 2013].

Beckman, K. & Ames, B., 1998. The free radical theory of aging matures. *Physiological reviews*. Available at: http://physrev.physiology.org/content/78/2/547.short [Accessed September 23, 2013].

Bedhomme, M. et al., 2005. Folate metabolism in plants: an Arabidopsis homolog of the mammalian mitochondrial folate transporter mediates folate import into chloroplasts. *The Journal of biological chemistry*, 280(41), pp.34823–31. Available at: http://www.jbc.org/content/280/41/34823.full [Accessed September 26, 2013].

Bik, E.M. et al., 2006. Molecular analysis of the bacterial microbiota in the human stomach. *Proceedings of the National Academy of Sciences of the United States of America*, 103(3), pp.732–7. Available at: http://www.pnas.org/content/103/3/732.short [Accessed September 23, 2013].

Björkman, A. & Phillips-Howard, P.A., 1991. Adverse reactions to sulfa drugs: implications for malaria chemotherapy. *Bulletin of the World Health Organization*, 69(3), pp.297–304. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2393107&tool=pmcentrez&rendertype=abstract [Accessed July 31, 2013].

Blatch, S.A., Meyer, K.W. & Harrison, J.F., 2010. Effects of dietary folic acid level and symbiotic folate production on fitness and development in the fruit fly

Drosophila melanogaster. *Fly*, 4(4), pp.312–9. Available at: https://www.landesbioscience.com/journals/fly/article/13258/ [Accessed September 1, 2013].

Brenner, S., 1974. THE GENETICS OF CAENORHABDITIS ELEGANS. *Genetics*, 77(1), pp.71–94. Available at: http://www.genetics.org/content/77/1/71.short [Accessed July 25, 2013].

Brown, J.P., Davidson, G.E. & Scott, J.M., 1974. The identification of the forms of folate found in the liver, kidney and intestine of the monkey and their biosynthesis from exogenous pteroylglutamate (folic acid). *Biochimica et Biophysica Acta (BBA) - General Subjects*, 343(1), pp.78–88. Available at: http://www.sciencedirect.com/science/article/pii/0304416574902402 [Accessed September 24, 2013].

Byerly, L., Cassada, R.C. & Russell, R.L., 1976. The life cycle of the nematode Caenorhabditis elegans. *Developmental Biology*, 51(1), pp.23–33. Available at: http://www.sciencedirect.com/science/article/pii/0012160676901196 [Accessed September 19, 2013].

Cabreiro, F. et al., 2013. Metformin retards aging in C. elegans by altering microbial folate and methionine metabolism. *Cell*, 153(1), pp.228–39. Available at: http://dx.doi.org/10.1016/j.cell.2013.02.035 [Accessed August 13, 2013].

Campisi, J. & Fagagna, F. di, 2007. Cellular senescence: when bad things happen to good cells. *Nature Reviews Molecular Cell Biology*. Available at: http://www.nature.com/nrm/journal/v8/n9/abs/nrm2233.html [Accessed September 23, 2013].

Cani, P. et al., 2007. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia*. Available at: http://link.springer.com/article/10.1007/s00125-007-0791-0 [Accessed September 23, 2013].

Cappuccio, F., 1997. Ethnicity and cardiovascular risk: variations in people of African ancestry and South Asian origin. *Journal of human hypertension*. Available at: http://www2.warwick.ac.uk/fac/med/staff/cappuccio/publications/jhumhypert97ethnicity.pdf [Accessed September 23, 2013].

Cappuccio, F., 1998. The Wandsworth Heart and Stroke Study. A population-based survey of cardiovascular risk factors in different ethnic groups. Methods and baseline. *NUTRITION* .... Available at: http://scholar.google.com/scholar?q=related:rnTFz8h_iDsJ:scholar.google.com/&hl=en&as_sdt=0,5#0 [Accessed September 23, 2013].

Carter, E.L. et al., 2007. Escherichia coli abg genes enable uptake and cleavage of the folate catabolite p-aminobenzoyl-glutamate. *Journal of bacteriology*, 189(9), pp.3329–34. Available at: http://jb.asm.org/content/189/9/3329.long [Accessed July 31, 2013].

Castelli, L., 2001. Sulfa drug screening in yeast: fifteen sulfa drugs compete with p-aminobenzoate in Saccharomyces cerevisiae. *FEMS Microbiology Letters*, 199(2), pp.181–184. Available at: http://dx.doi.org/10.1016/S0378-1097(01)00172-0 [Accessed July 31, 2013].

Cech, T., 2004. Beginning to understand the end of the chromosome. *Cell*. Available at: http://www.sciencedirect.com/science/article/pii/S0092867404000388 [Accessed September 23, 2013].

Chabner, B.A. et al., 1985. Polyglutamation of methotrexate. Is methotrexate a prodrug? *The Journal of clinical investigation*, 76(3), pp.907–12. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=423952&tool=pmcentrez&rendertype=abstract [Accessed September 5, 2013].

Chan, E.S.L. & Cronstein, B.N., 2002. Molecular action of methotrexate in inflammatory diseases. *Arthritis Research Vol 4 No 4*, pp.266–273. Available at: http://www.biomedcentral.com/content/pdf/ar419.pdf [Accessed September 5, 2013].

Charlesworth, B. & Hughes, K., 1996. Age-specific inbreeding depression and components of genetic variance in relation to the evolution of senescence. *Proceedings of the National* .... Available at: http://www.pnas.org/content/93/12/6140.short [Accessed March 30, 2014].

Chow, D.K. et al., 2006. Sarcopenia in the Caenorhabditis elegans pharynx correlates with muscle contraction rate over lifespan. *Experimental gerontology*, 41(3), pp.252–60. Available at: http://dx.doi.org/10.1016/j.exger.2005.12.004 [Accessed September 11, 2013].

Connor, E.E., 1998. Sulfonamide antibiotics. *Primary Care Update for OB/GYNS*, 5(1), pp.32–35. Available at: http://dx.doi.org/10.1016/S1068-607X(97)00121-2 [Accessed July 31, 2013].

Curtis, H., 1963. Biological mechanisms underlying the aging process. *Science See Saiensu*. Available at: http://www.osti.gov/energycitations/product.biblio.jsp?osti_id=4683491 [Accessed September 23, 2013].

Dodd, M.C., 2004. Transformation of the Antibacterial Agent Sulfamethoxazole in Reactions with Chlorine : Kinetics , Mechanisms , and Pathways. , 38(21), pp.5607–5615.

Domagk, G., 1957. TWENTY-FIVE YEARS OF SULFONAMIDE THERAPY. *Annals of the New York Academy of Sciences*, 69(3), pp.380–384. Available at: http://doi.wiley.com/10.1111/j.1749-6632.1957.tb49674.x [Accessed July 31, 2013].

Dunne, C., 2001. Adaptation of Bacteria to the Intestinal Niche: Probiotics and Gut Disorder. *Inflammatory Bowel Diseases*, 7(2), pp.136–145. Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00054725-200105000-00010 [Accessed September 23, 2013].

El- Ami, T., Moll, L. & Marques, F.C., 2014. A novel inhibitor of the insulin/IGF signaling pathway protects from age- onset, neurodegeneration- linked proteotoxicity. *Aging cell*. Available at: http://onlinelibrary.wiley.com/doi/10.1111/acel.12171/full [Accessed March 30, 2014].

Fei, N. & Zhao, L., 2013. An opportunistic pathogen isolated from the gut of an obese human causes obesity in germfree mice. *The ISME journal*, 7(4), pp.880–4. Available at: http://dx.doi.org/10.1038/ismej.2012.153 [Accessed May 27, 2014].

Félix, M.-A. & Braendle, C., 2010. The natural history of Caenorhabditis elegans. *Current Biology*, 20(22), pp.R965–R969. Available at: http://www.sciencedirect.com/science/article/pii/S0960982210011681 [Accessed September 24, 2013].

Ferone, R., 1977. Folate metabolism in malaria. *Bulletin of the World Health Organization*, 55(2-3), pp.291–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2366725&tool=pmcentrez&rendertype=abstract [Accessed July 31, 2013].

Finch, C., 1994. *Longevity, senescence, and the genome*, Available at: http://books.google.co.uk/books?hl=en&lr=&id=_JkMRundeNcC&oi=fnd&pg=PR11&dq=Finch,+C.+E.+Longevity,+Senescence+and+the+Genome&ots=fn1j2aaWkO&sig=VXQCEbE63GUjNoxYsZRyZeW9CE4 [Accessed September 23, 2013].

Fischi, M., Dickinson, G. & Vole, L. La, 1988. Safety and efficacy of sulfamethoxazole and trimethoprim chemoprophylaxis for Pneumocystis carinii pneumonia in AIDS. *JAMA: the journal of the* .... Available at: http://jama.ama-assn.org/content/259/8/1185.short [Accessed September 26, 2013].

Francis, R. & Waterston, R., 1991. Muscle cell attachment in Caenorhabditis elegans. *The Journal of cell biology*. Available at: http://jcb.rupress.org/content/114/3/465.abstract [Accessed March 30, 2014].

Friedman, D.B. & Johnson, T.E., 2002. A Mutation in the age-1 Gene in Caenorhabditis elegans Lengthens Life and Reduces Hermaphrodite Fertility. *Science of Aging Knowledge Environment*, 2002(38), p.22. Available at:

http://sageke.sciencemag.org/cgi/content/abstract/2002/38/cp22 [Accessed March 30, 2014].

Garigan, D. et al., 2002. Genetic analysis of tissue aging in Caenorhabditis elegans: a role for heat-shock factor and bacterial proliferation. *Genetics*, 161(3), pp.1101–12. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1462187&tool=pmcentrez&rendertype=abstract.

Garratt, L.C. et al., 2005. Comprehensive metabolic profiling of mono- and polyglutamated folates and their precursors in plant and animal tissue using liquid chromatography/negative ion electrospray ionisation tandem mass spectrometry. *Rapid communications in mass spectrometry : RCM*, 19(17), pp.2390–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16047318 [Accessed July 25, 2013].

Gems, D. & Riddle, D.L., 2000. Genetic, Behavioral and Environmental Determinants of Male Longevity in Caenorhabditis elegans. *Genetics*, 154(4), pp.1597–1610. Available at: http://www.genetics.org/content/154/4/1597.short [Accessed September 11, 2013].

Gill, H. & Guarner, F., 2004. Probiotics and human health: a clinical perspective. *Postgraduate Medical Journal*. Available at: http://pmj.bmj.com/content/80/947/516.short [Accessed March 30, 2014].

Gomez, F. et al., 2012. Delayed accumulation of intestinal coliform bacteria enhances life span and stress resistance in Caenorhabditis elegans fed respiratory deficient

E. coli. *BMC microbiology*, 12(1), p.300. Available at: http://www.biomedcentral.com/1471-2180/12/300 [Accessed August 8, 2013].

Green, J., Nichols, B. & Matthews, R., 1996. Folate biosynthesis, reduction, and polyglutamylation. *Escherichia coli and Salmonella: cellular and* .... Available at: http://scholar.google.co.uk/scholar?hl=en&q=green+Folate+Biosynthesis,+Reduction+and+Polyglutamylation&btnG=&as_sdt=1,5&as_sdtp=#0 [Accessed September 26, 2013].

Greer, E. et al., 2007. An AMPK-FOXO Pathway Mediates Longevity Induced by a Novel Method of Dietary Restriction in< i> C. elegans</i>. *Current biology*. Available at: http://www.sciencedirect.com/science/article/pii/S0960982207018647 [Accessed March 30, 2014].

Greer, E.L. & Brunet, A., 2009. Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in C. elegans. *Aging cell*, 8(2), pp.113–27. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2680339&tool=pmcentrez&rendertype=abstract [Accessed May 22, 2013].

Guarner, F. & Malagelada, J.-R., 2003. Gut flora in health and disease. *Lancet*, 361(9356), pp.512–9. Available at: http://www.sciencedirect.com/science/article/pii/S0140673603124890 [Accessed May 25, 2014].

Guarner, F. & Schaafsma, G., 1998. Probiotics. *International journal of food microbiology*. Available at: http://scholar.google.co.uk/scholar?q=probiotics+guarner&btnG=&hl=en&as_sdt=0,5#0 [Accessed May 2, 2014].

HACKMANN, C., 1958. [Observations on influenceability of age phenomena in experimental animals by peroral administration of combinations of 2-(p-aminobenzolsulfonamide)-pyrimidin]. *Münchener medizinische Wochenschrift (1950)*, 100(47), pp.1814–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13622503 [Accessed September 27, 2013].

Halsted, C.H., 1998. Folylpoly-gamma -glutamate Carboxypeptidase from Pig Jejunum. MOLECULAR CHARACTERIZATION AND RELATION TO GLUTAMATE CARBOXYPEPTIDASE II. *Journal of Biological Chemistry*, 273(32), pp.20417–20424. Available at: http://www.jbc.org/content/273/32/20417.short [Accessed September 5, 2013].

Hansen, M. et al., 2008. A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. *PLoS genetics*, 4(2), p.e24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2242811&tool=pmcentrez&rendertype=abstract [Accessed June 6, 2013].

Hansen, M. et al., 2005. New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a Caenorhabditis elegans genomic RNAi screen. *PLoS genetics*. Available at: http://dx.plos.org/10.1371/journal.pgen.0010017 [Accessed September 26, 2013].

Harley, C., 1991. Telomere loss: mitotic clock or genetic time bomb? *Mutation Research/DNAging*. Available at: http://www.sciencedirect.com/science/article/pii/0921873491900187 [Accessed September 23, 2013].

Harley, C. & Vaziri, H., 1992. The telomere hypothesis of cellular aging. *Experimental gerontology*. Available at: http://scholar.google.co.uk/scholar?hl=en&q=telomere+loss+theory+ageing&btnG=&as_sdt=1,5&as_sdtp=#8 [Accessed September 23, 2013].

Harman, D., 1992. Free radical theory of aging. *Mutation Research/DNAging*. Available at: http://www.sciencedirect.com/science/article/pii/092187349290030S [Accessed September 23, 2013].

Harman, D., 1993. Free radical theory of aging. *Free Radicals: from Basic Science to Medicine*. Available at: http://link.springer.com/chapter/10.1007/978-3-0348-9116-5_11 [Accessed September 23, 2013].

Hayflick, L., 1965. The limited< i> in vitro</i> lifetime of human diploid cell strains. *Experimental cell research*. Available at: http://www.sciencedirect.com/science/article/pii/0014482765902119 [Accessed September 23, 2013].

Herbig, U. et al., 2006. Cellular senescence in aging primates. *Science*. Available at: http://www.sciencemag.org/content/311/5765/1257.short [Accessed May 3, 2014].

Herndon, L.A. et al., 2002. Stochastic and genetic factors influence tissue-specific decline in ageing C. elegans. *Nature*, 419(6909), pp.808–14. Available at: http://www.nature.com/nature/journal/v419/n6909/pdf/nature01135.pdf [Accessed August 31, 2013].

Hjortmo, S., Patring, J. & Andlid, T., 2008. Growth rate and medium composition strongly affect folate content in Saccharomyces cerevisiae. *International journal of food microbiology*, 123(1-2), pp.93–100. Available at: http://dx.doi.org/10.1016/j.ijfoodmicro.2007.12.004 [Accessed August 19, 2013].

Hoffbrand, A. V. et al., 1971. Small intestinal bacterial flora and folate status in gastrointestinal disease. *Gut*, 12(1), pp.27–33. Available at: http://gut.bmj.com/content/12/1/27.abstract [Accessed September 26, 2013].

Hooper, L. et al., 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science*. Available at: http://www.sciencemag.org/content/291/5505/881.short [Accessed May 3, 2014].

Hooper, L., Midtvedt, T. & Gordon, J., 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual review of nutrition*. Available at: http://www.annualreviews.org/doi/abs/10.1146/annurev.nutr.22.011602.092259 [Accessed May 3, 2014].

Huang, M. & Pittard, J., 1967. Genetic Analysis of Mutant Strains of Escherichia coli Requiring p-Aminobenzoic Acid for Growth. *J. Bacteriol.*, 93(6), pp.1938–1942. Available at:

http://jb.asm.org/content/93/6/1938.abstract?ijkey=ec4e8c50b44f54724af14a92bba7dd0859a46c61&keytype2=tf_ipsecsha [Accessed July 31, 2013].

Hughes, K. & Charlesworth, B., 1994. A genetic analysis of senescence in Drosophila. *Nature*. Available at: http://www.life.illinois.edu/kahughes/Hughes&Charlesworth94.pdf [Accessed May 3, 2014].

Iwai, K., Kobashi, M. & Fujisawa, H., 1970. Occurrence of Crithidia factors and folic acid in various bacteria. *Journal of bacteriology*. Available at: http://jb.asm.org/content/104/1/197.short [Accessed September 26, 2013].

Jeyapalan, J. & Ferreira, M., 2007. Accumulation of senescent cells in mitotic tissue of aging primates. *Mechanisms of ageing and* .... Available at: http://www.sciencedirect.com/science/article/pii/S0047637406002417 [Accessed March 30, 2014].

Kamath, R. & Ahringer, J., 2003. Genome-wide RNAi screening in< i> Caenorhabditis elegans</i>. *Methods*. Available at: http://www.sciencedirect.com/science/article/pii/S1046202303000501 [Accessed March 30, 2014].

Kenyon, C. et al., 1993. A C. elegans mutant that lives twice as long as wild type. *Nature*. Available at: http://nemates.org/uky/425/Kenyon.1993.Nature.v366.p461-4.pdf [Accessed March 30, 2014].

Kim, T. & Yang, J., 2004. A large pool of available folate exists in the large intestine of human infants and piglets. *The Journal of nutrition*. Available at: http://jn.nutrition.org/content/134/6/1389.short [Accessed September 24, 2013].

Kirkwood, T., 1977. Evolution of ageing. *Nature*. Available at: http://europepmc.org/abstract/MED/593350 [Accessed September 23, 2013].

Kirkwood, T., 2008. Understanding ageing from an evolutionary perspective. *Journal of internal medicine*. Available at: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2796.2007.01901.x/full [Accessed May 20, 2014].

Kirkwood, T. & Holliday, R., 1979. The evolution of ageing and longevity. ... *of the Royal* .... Available at: http://rspb.royalsocietypublishing.org/content/205/1161/531.short [Accessed September 23, 2013].

Kirkwood, T.B. & Austad, S.N., 2000. Why do we age? *Nature*, 408(6809), pp.233–8. Available at: http://dx.doi.org/10.1038/35041682 [Accessed September 19, 2013].

Kirkwood, T.B.L., 2005. Understanding the Odd Science of Aging. *Cell*, 120(4), pp.437–447. Available at: http://www.sciencedirect.com/science/article/pii/S0092867405001017 [Accessed September 19, 2013].

Kirkwood, T.B.L. & Melov, S., 2011. On the programmed/non-programmed nature of ageing within the life history. *Current biology : CB*, 21(18), pp.R701–7.

Available at: http://www.sciencedirect.com/science/article/pii/S0960982211007895 [Accessed May 15, 2014].

Kobayashi, Y. et al., 1978. Treatment of seven cases of chronic granulomatous disease with sulfamethoxazole-trimethoprim (SMX-TMP). *European Journal of Pediatrics*, 127(4), pp.247–254. Available at: http://link.springer.com/10.1007/BF00493540 [Accessed September 1, 2013].

Krtolica, A. & Campisi, J., 2002. Cancer and aging: a model for the cancer promoting effects of the aging stroma. *The international journal of biochemistry & cell ...*. Available at: http://www.sciencedirect.com/science/article/pii/S1357272502000535 [Accessed May 3, 2014].

Kwon, Y.K. et al., 2008a. A domino effect in antifolate drug action in Escherichia coli. *Nature chemical biology*, 4(10), pp.602–8. Available at: http://dx.doi.org/10.1038/nchembio.108 [Accessed August 10, 2013].

Kwon, Y.K. et al., 2008b. A domino effect in antifolate drug action in Escherichia coli. *Nature chemical biology*, 4(10), pp.602–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2699209&tool=pmcentrez&rendertype=abstract [Accessed June 17, 2013].

Larsen, N. & Vogensen, F., 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PloS one*. Available at: http://dx.plos.org/10.1371/journal.pone.0009085 [Accessed September 23, 2013].

Lenaerts, I. et al., 2008. Dietary restriction of Caenorhabditis elegans by axenic culture reflects nutritional requirement for constituents provided by metabolically active microbes. *The journals of gerontology Series A Biological sciences and medical sciences*, 63(3), pp.242–252. Available at: http://discovery.ucl.ac.uk/49742/.

Leoung, G.S. et al., 2001. Trimethoprim-sulfamethoxazole (TMP-SMZ) dose escalation versus direct rechallenge for Pneumocystis Carinii pneumonia prophylaxis in human immunodeficiency virus-infected patients with previous adverse reaction to TMP-SMZ. *The Journal of infectious diseases*, 184(8), pp.992–7. Available at: http://jid.oxfordjournals.org/content/184/8/992.full [Accessed September 26, 2013].

Leroi, A.M. et al., 2005. What evidence is there for the existence of individual genes with antagonistic pleiotropic effects? *Mechanisms of ageing and development*, 126(3), pp.421–9. Available at: http://www.sciencedirect.com/science/article/pii/S0047637404001897 [Accessed May 8, 2014].

Levy, M., Allsopp, R. & Futcher, A., 1992. Telomere end-replication problem and cell aging. *Journal of molecular* .... Available at: http://www.sciencedirect.com/science/article/pii/0022283692900963 [Accessed September 23, 2013].

Ley, R. et al., 2006. Microbial ecology: human gut microbes associated with obesity. *Nature*. Available at: http://europepmc.org/abstract/MED/17183309 [Accessed September 23, 2013].

Li, M. & Wang, B., 2008. Symbiotic gut microbes modulate human metabolic phenotypes. *Proceedings of the* .... Available at: http://www.pnas.org/content/105/6/2117.short [Accessed September 23, 2013].

Lu, W., Kwon, Y.K. & Rabinowitz, J.D., 2007. Isotope ratio-based profiling of microbial folates. *Journal of the American Society for Mass Spectrometry*, 18(5), pp.898–909. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1909916&tool=pmcentrez&rendertype=abstract [Accessed July 25, 2013].

Lyon, C.J. et al., 2000. The C. elegans apoptotic nuclease NUC-1 is related in sequence and activity to mammalian DNase II. *Gene*, 252(1-2), pp.147–154. Available at: http://dx.doi.org/10.1016/S0378-1119(00)00213-4 [Accessed September 5, 2013].

Mair, W., Piper, M. & Partridge, L., 2005. Calories do not explain extension of life span by dietary restriction in Drosophila. *PLoS biology*. Available at: http://dx.plos.org/10.1371/journal.pbio.0030223.g005 [Accessed May 3, 2014].

Man, J. De, 1960. A medium for the cultivation of lactobacilli. *Journal of Applied* .... Available at: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2672.1960.tb00188.x/full [Accessed September 26, 2013].

Mattison, J., 2003. Calorie restriction in rhesus monkeys. *Experimental Gerontology*, 38(1-2), pp.35–46. Available at: http://www.sciencedirect.com/science/article/pii/S0531556502001468 [Accessed May 10, 2014].

McGee, M.D. et al., 2011. Loss of intestinal nuclei and intestinal integrity in aging C. elegans. *Aging cell*, 10(4), pp.699–710. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3135675&tool=pmcentrez&rendertype=abstract [Accessed August 28, 2013].

McKay, J., 2004. Folate and DNA methylation during in utero development and aging. *Biochemical Society* .... Available at: http://www-06.all-portland.net/bst/032/1006/0321006.pdf [Accessed September 24, 2013].

Medawar, P., 1952. An unsolved problem of biology. Available at: http://scholar.google.co.uk/scholar?q=Medawar,+P.+B.+An+Unsolved+Problem+of+Biology+(Lewis,+London,+1952).&btnG=&hl=en&as_sdt=0,5#0 [Accessed September 23, 2013].

Medvedev, Z., 1990. An attempt at a rational classification of theories of ageing. *Biological Reviews*. Available at: http://onlinelibrary.wiley.com/doi/10.1111/j.1469-185X.1990.tb01428.x/full [Accessed September 23, 2013].

Mello, C.C. et al., 1991. Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. *The EMBO journal*, 10(12), pp.3959–70. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=453137&tool=pmcentrez&rendertype=abstract [Accessed August 30, 2013].

Miyazawa, E., Iwabuchi, A. & Yoshida, T., 1996. Phytate breakdown and apparent absorption of phosphorus, calcium and magnesium in germfree and conventionalized rats. *Nutrition research*. Available at:

http://www.sciencedirect.com/science/article/pii/0271531796000383 [Accessed May 3, 2014].

Moore, W. & Moore, L., 1995. Intestinal floras of populations that have a high risk of colon cancer. *Applied and Environmental Microbiology*. Available at: http://aem.asm.org/content/61/9/3202.short [Accessed May 2, 2014].

Musso, G., Gambino, R. & Cassader, M., 2010. Obesity, Diabetes, and Gut Microbiota The hygiene hypothesis expanded? *Diabetes care*. Available at: http://care.diabetesjournals.org/content/33/10/2277.short [Accessed September 23, 2013].

Nguyen, T.P.T. & Clarke, C.F., 2012. Folate status of gut microbiome affects Caenorhabditis elegans lifespan. *BMC biology*, 10(1), p.66. Available at: http://www.biomedcentral.com/1741-7007/10/66 [Accessed July 31, 2013].

Olovnikov, A., 1996. Telomeres, telomerase, and aging: origin of the theory. *Experimental gerontology*. Available at: http://www.sciencedirect.com/science/article/pii/0531556596000058 [Accessed September 23, 2013].

Olsen, A., Vantipalli, M.C. & Lithgow, G.J., 2006. Using Caenorhabditis elegans as a model for aging and age-related diseases. *Annals of the New York Academy of Sciences*, 1067, pp.120–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16803977 [Accessed May 3, 2014].

Patel, O.G. et al., 2004. Sulfa drugs strike more than once. *Trends in Parasitology*, 20(1), pp.1–3. Available at:

http://www.sciencedirect.com/science/article/pii/S1471492203002824 [Accessed September 25, 2013].

Pham, P., Perret, S. & Cass, B., 2005. Transient gene expression in HEK293 cells: peptone addition posttransfection improves recombinant protein synthesis. *Biotechnology and* .... Available at: http://onlinelibrary.wiley.com/doi/10.1002/bit.20428/full [Accessed September 26, 2013].

Pincus, Z. & Slack, F.J., 2010. Developmental biomarkers of aging in Caenorhabditis elegans. *Developmental dynamics : an official publication of the American Association of Anatomists*, 239(5), pp.1306–14. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3073312&tool=pmcentrez&rendertype=abstract [Accessed August 15, 2013].

Pompei, A. & Cordisco, L., 2007. Folate production by bifidobacteria as a potential probiotic property. *Applied and* .... Available at: http://aem.asm.org/content/73/1/179.short [Accessed September 26, 2013].

Portal-Celhay, C., Bradley, E.R. & Blaser, M.J., 2012. Control of intestinal bacterial proliferation in regulation of lifespan in Caenorhabditis elegans. *BMC microbiology*, 12(1), p.49. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3342110&tool=pmcentrez&rendertype=abstract [Accessed July 5, 2013].

Promislow, D. & Tatar, M., 1996. Age-specific patterns of genetic variance in Drosophila melanogaster. I. Mortality. *Genetics*. Available at: http://www.genetics.org/content/143/2/839.short [Accessed May 2, 2014].

Qin, J. et al., 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, 464(7285), pp.59–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20203603 [Accessed May 21, 2013].

Reboul, J. et al., 2001. Open-reading-frame sequence tags (OSTs) support the existence of at least 17,300 genes in C. elegans. *Nature genetics*, 27(3), pp.332–6. Available at: http://dx.doi.org/10.1038/85913 [Accessed May 1, 2014].

Reed, M.C. et al., 2006. A Mathematical Model Gives Insights into Nutritional and Genetic Aspects of Folate-Mediated One-Carbon Metabolism. *J. Nutr.*, 136(10), pp.2653–2661. Available at: http://jn.nutrition.org/content/136/10/2653.short [Accessed September 23, 2013].

Reinke, V., 2002. Functional exploration of the C. elegans genome using DNA microarrays. *nature genetics*. Available at: http://www.nature.com/ng/journal/v32/n4s/full/ng1039.html [Accessed September 23, 2013].

Roland, S. et al., 1979. The Characteristics and Significance of Sulfonamides as Substrates for Escherichia coli Dihydropteroate Synthase. *Journal of biological chemistry*, 254, pp.10337–10345. Available at: http://www.jbc.org/content/254/20/10337.full.pdf [Accessed July 31, 2013].

Rossi, M., Amaretti, A. & Raimondi, S., 2011. Folate production by probiotic bacteria. *Nutrients*. Available at: http://www.mdpi.com/2072-6643/3/1/118 [Accessed September 26, 2013].

Rubin, H., 2002. The disparity between human cell senescence in vitro and lifelong replication in vivo. *Nature biotechnology*. Available at: http://www.nature.com/nbt/journal/v20/n7/abs/nbt0702-675.html [Accessed March 30, 2014].

Ruskin, J. & LaRiviere, M., 1991. Low-dose co-trimoxazole for prevention of Pneumocystis carinii pneumonia in human immunodeficiency virus disease. *The Lancet*. Available at: http://www.sciencedirect.com/science/article/pii/014067369193402U [Accessed September 26, 2013].

Saiki, R. et al., 2008. Altered bacterial metabolism, not coenzyme Q content, is responsible for the lifespan extension in Caenorhabditis elegans fed an Escherichia coli diet lacking coenzyme Q. *Aging cell*, 7(3), pp.291–304. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3104051&tool=pmcentrez&rendertype=abstract [Accessed May 23, 2013].

Scott, J., R&#xfffd;beill&#xfffd;, F. & Fletcher, J., 2000. Folic acid and folates: the feasibility for nutritional enhancement in plant foods. *Journal of the Science of Food and Agriculture*, 80(7), pp.795–824. Available at: http://doi.wiley.com/10.1002/(SICI)1097-0010(20000515)80:7<795::AID-JSFA599>3.0.CO;2-K [Accessed August 29, 2013].

Scozzafava, A. et al., 2003. Anticancer and Antiviral Sulfonamides. *Current Medicinal Chemistry*, 10(11), pp.925–953. Available at:

http://www.ingentaconnect.com/content/ben/cmc/2003/00000010/00000011/art00003 [Accessed July 31, 2013].

Service, P., Hutchinson, E. & Rose, M., 1988. Multiple genetic mechanisms for the evolution of senescence in Drosophila melanogaster. *Evolution*. Available at: http://www.jstor.org/stable/2408862 [Accessed March 30, 2014].

Shane, B. & Stokstad, E.L., 1975. Transport and metabolism of folates by bacteria. *J. Biol. Chem.*, 250(6), pp.2243–2253. Available at: http://www.jbc.org/content/250/6/2243.abstract?ijkey=f78f57e8e46506cfb388f570f83850332cda211d&keytype2=tf_ipsecsha [Accessed July 31, 2013].

Shane, B. & Stokstad, E.L.R., 1985. VITAMIN B12-FOLATE.

Shaw, F., Promislow, D. & Tatar, M., 1999. Toward reconciling inferences concerning genetic variation in senescence in Drosophila melanogaster. …. Available at: http://www.genetics.org/content/152/2/553.short [Accessed March 30, 2014].

Simon, G. & Gorbach, S., 1986. The human intestinal microflora. *Digestive diseases and sciences*. Available at: http://link.springer.com/article/10.1007/BF01295996 [Accessed March 30, 2014].

Sonnhammer, E. & Durbin, R., 1997. Analysis of Protein Domain Families in< i> Caenorhabditis elegans</i>. *Genomics*. Available at: http://www.sciencedirect.com/science/article/pii/S0888754397949892 [Accessed February 3, 2014].

Stanisich, V.A., Bennett, P.M. & Ortiz, J.M., 1976. A molecular analysis of transductional marker rescue involving P-group plasmids in Pseudomonas aeruginosa. *MGG Molecular & General Genetics*, 143(3), pp.333–337. Available at: http://link.springer.com/10.1007/BF00269412 [Accessed August 1, 2013].

Stokstad, E.L.R. & Jukes, T.H., 1987. Sulfonamides and Folic Acid Antagonists □: A Historical Review. *The Journal of nutrition*, (December 1986), pp.1335–1341.

Stover, P. & Field, M., 2011. Trafficking of intracellular folates. ... *in Nutrition: An International Review Journal*. Available at: http://advances.nutrition.org/content/2/4/325.short [Accessed September 24, 2013].

Supuran, C.T., 2003. Indisulam: an anticancer sulfonamide in clinical development. *Expert opinion on investigational drugs*, 12(2), pp.283–7. Available at: http://informahealthcare.com/doi/abs/10.1517/13543784.12.2.283 [Accessed September 26, 2013].

Szewczyk, N.J. et al., 2006. Delayed development and lifespan extension as features of metabolic lifestyle alteration in C. elegans under dietary restriction. *The Journal of experimental biology*, 209(Pt 20), pp.4129–39. Available at: http://jeb.biologists.org/content/209/20/4129.short [Accessed August 15, 2013].

Timmons, L., Court, D.L. & Fire, A., 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene*, 263(1-2), pp.103–112. Available at: http://dx.doi.org/10.1016/S0378-1119(00)00579-5 [Accessed July 31, 2013].

Torok-Storb, B., 1988. Cellular interactions. *Blood*. Available at: http://bloodjournal.hematologylibrary.org/content/72/2/373.short [Accessed May 30, 2014].

Turnbaugh, P.J. et al., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(7122), pp.1027–31. Available at: http://dx.doi.org/10.1038/nature05414 [Accessed September 22, 2013].

Valdivia, R. & Falkow, S., 1996. Bacterial genetics by flow cytometry: rapid isolation of Salmonella typhimurium acid-inducible promoters by differential fluorescence induction. *Molecular microbiology*. Available at: http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2958.1996.00120.x/full [Accessed May 27, 2014].

Vijg, J., 2000. Somatic mutations and aging: a re-evaluation. *Mutation Research/Fundamental and Molecular* .... Available at: http://www.sciencedirect.com/science/article/pii/S002751079900202X [Accessed September 23, 2013].

Villarroel, R. & Hedges, R., 1983. Heteroduplex analysis of P-plasmid evolution: the role of insertion and deletion of transposable elements. *Molecular and General* .... Available at: http://link.springer.com/article/10.1007/BF00325900 [Accessed May 27, 2014].

Virk, B. et al., 2012. Excessive folate synthesis limits lifespan in the C. elegans: E. coli aging model. *BMC biology*, 10(1), p.67. Available at: http://www.biomedcentral.com/1741-7007/10/67 [Accessed August 21, 2013].

Wakayama, T., Shinkai, Y. & Tamashiro, K., 2000. Ageing: cloning of mice to six generations. *Nature*. Available at: http://www.nature.com/nature/journal/v407/n6802/abs/407318b0.html [Accessed September 27, 2013].

Walker, G. & Houthoofd, K., 2005. Dietary restriction in< i> C. elegans</i>: From rate-of-living effects to nutrient sensing pathways. *Mechanisms of ageing ....* Available at: http://www.sciencedirect.com/science/article/pii/S0047637405000801 [Accessed September 24, 2013].

Wang, Z. et al., 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. Available at: http://www.nature.com/nature/journal/v472/n7341/abs/nature09922.html [Accessed September 23, 2013].

Ward, S. & Thomson, N., 1975. Electron microscopical reconstruction of the anterior sensory anatomy of the nematode Caenorhabditis elegans. *Journal of Comparative ....* Available at: http://onlinelibrary.wiley.com/doi/10.1002/cne.901600305/full [Accessed May 11, 2014].

Weening, R.S. et al., 1983. Continuous therapy with sulfamethoxazole-trimethoprim in patients with chronic granulomatous disease. *The Journal of pediatrics*, 103(1), pp.127–30. Available at: http://www.jpeds.com/article/S0022-3476(83)80798-7/abstract [Accessed September 1, 2013].

Weindruch, R. et al., 2002. Gene expression profiling of aging using DNA microarrays. *Mechanisms of ageing and* .... Available at: http://www.sciencedirect.com/science/article/pii/S004763740100344X [Accessed September 27, 2013].

Weindruch, R. et al., 2001. Microarray profiling of gene expression in aging and its alteration by caloric restriction in mice. *The Journal of nutrition*. Available at: http://jn.nutrition.org/content/131/3/918S.short [Accessed September 27, 2013].

Weinert, B. & Timiras, P., 2003. Invited review: theories of aging. *Journal of Applied Physiology*. Available at: http://jap.physiology.org/content/95/4/1706.short [Accessed September 23, 2013].

Wen, L., Ley, R. & Volchkov, P., 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature*. Available at: http://www.nature.com/nature/journal/vaop/ncurrent/full/nature07336.html [Accessed September 23, 2013].

Whittle, S.L. & Hughes, R.A., 2004. Folate supplementation and methotrexate treatment in rheumatoid arthritis: a review. *Rheumatology (Oxford, England)*, 43(3), pp.267–71. Available at: http://rheumatology.oxfordjournals.org/content/43/3/267.short [Accessed August 28, 2013].

Willcox, B., Donlon, T. & He, Q., 2008. FOXO3A genotype is strongly associated with human longevity. *Proceedings of the* .... Available at: http://www.pnas.org/content/105/37/13987.short [Accessed March 30, 2014].

Williams, B., Schrank, B. & Huynh, C., 1992. A genetic mapping system in Caenorhabditis elegans based on polymorphic sequence-tagged sites. …. Available at: http://www.genetics.org/content/131/3/609.short [Accessed May 2, 2014].

Williams, G., 2001. Pleiotropy, natural selection, and the evolution of senescence. *Science's SAGE KE*. Available at: http://sageke.sciencemag.org/cgi/content/abstract/2001/1/cp13 [Accessed September 23, 2013].

Yamamoto, N., 2009. Update on the Keio collection of Escherichia coli single-gene deletion mutants. *Molecular systems* .... Available at: http://onlinelibrary.wiley.com/doi/10.1038/msb.2009.92/full [Accessed May 2, 2014].

Yatsunenko, T. et al., 2012. Human gut microbiome viewed across age and geography. *Nature*, 486(7402), pp.222–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3376388&tool=pmcentrez&rendertype=abstract [Accessed May 21, 2013].

Yeldandi, V., Strodtman, R. & Lentino, J.R., 1988. In-vitro and in-vivo studies of trimethoprim-sulphamethoxazole against multiple resistant Staphylococcus aureus. *Journal of Antimicrobial Chemotherapy*, 22(6), pp.873–880. Available at: http://jac.oxfordjournals.org/content/22/6/873.abstract [Accessed September 1, 2013].

Younes, H. & Coudray, C., 2001. Effects of two fermentable carbohydrates (inulin and resistant starch) and their combination on calcium and magnesium balance in

rats. *British Journal of* .... Available at: http://journals.cambridge.org/abstract_S0007114501002161 [Accessed May 3, 2014].