How Zinc Transporters in Escherichia coli Influence Ageing in the Nematode Caenorhabditis elegans

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How Zinc Transporters in *Escherichia coli* Influence Ageing in the Nematode *Caenorhabditis elegans*

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ABSTRACT

Gut microbes play an important role in mammalian physiology. *Escherichia coli* not only provide the *Caenorhabditis elegans* with vital nutrients but also influence worms lifespan. Studying such interactions could help us to understand how intestinal microbes influence mammalian ageing. A recent gene deletion study of 1041 *E. coli* in our lab identified 9 genes that are involved in the increase of worm's lifespan. One gene identified was *ZnuB,* which forms part of the high affinity *znuABC* zinc ABC transporters, plays an important role in zinc homeostasis, and has been suggested to play a role in increased lifespan. To validate this hypothesis, levels of zinc were measured using ICP-MS in LB and NGM media, in *znuA*, *znuB*, and *znuC* mutant bacterial strains and worms fed with them, and compared with zinc levels in WT bacteria and *C. elegans* fed with them.

The results show that although bacteria and worms obtain zinc from LB media, the level of zinc was lower in worms and the three mutant bacteria than in WT bacteria alone. Subsequently, the lifespan of worms fed with the mutant bacterial strains was investigated. Worms fed with *znuB* and *znuC* bacteria showed extended lifespan as compared to worms fed with *znuA* bacteria. Reduced fecundity was observed in worms fed with mutant bacteria as compared to worms fed with WT bacteria. Moreover, the worms fed with *znuB* mutants showed a delay in the onset of egg laying. These results suggest that reducing zinc concentration itself in the mutant bacteria does not make the worms live longer, but the mutation in the *znuB* could produce different effects. Results of zinc supplementation experiments using *znuB* mutants showed a reversal of the effect on worm developmental delay. These results show that the *znuB* not only plays an important role in zinc uptake by bacteria, but also affects the lifespan of *C. elegans*.

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DECLARATION

I, Razan Bakheet, would like to declare that all the material of this study is solely my own work that has been performed without any aid. This work had not been submitted previously at any academic or professional level. The views represented in this study are my own and not those associated with other university.

CHAPTER 1: INTRODUCTION

1.1 Overview: Gut Microbes and Ageing

1.1.1 Gut Microbes

A large number of microorganisms that comprise various groups of bacteria are present in the digestive tract of all animals, including humans, and are considered as normal gut flora of the body. The important function of most of the gut microbes is to facilitate the digestion of the undigested complex carbohydrates and cellulose to release energy in the absence of major digestive enzymes. This released energy can be utilised by both the host and the microbe (Ley et al., 2008; Tremaroli and Backhed, 2012). Many vital functions of the short-chain fatty acids and other end products of the digestive actions of the microbes is to provide energy, and to act as modulators of inflammation, vascular function, motility and tissue repair to both local and peripheral tissue (Bergman, 1990).

There are approximately 100 trillion microorganisms present in the intestine of the humans, greater than the number of cells in the human body. It has been suggested that the gut microbiome complements human genome with approximately 100-fold more genes that are unique (Ley et al., 2006). Approximately, 1000 different types of microbe species are present in the gut (Round et al., 2010). Earlier studies have shown a symbiotic association between the normal microbe flora and the human body, and explained how they can remain harmless to each other (Round and Mazmanian, 2009). The functions of the micro-organisms include fermentation of different substances present in the body, inhibiting the growth of the pathogenic bacteria, educating and inducing innate immunity, production of hormone factors that affect energy metabolism

and storage of fats (e.g. fasting-induced adipocyte factor), regulating the gut physiology and production of essential vitamins (Tremaroli and Backhed, 2012).

1.1.2 Ageing

In humans, ageing is defined by progressive loss and decrease of both biological and physiological functions, decreasing mortality and declining fertility with advancing age (Kirkwood and Austad, 2000). Biological and chronological ageing are the two types of ageing implied in this definition. In the above definition, 'advancing age' of an individual is the chronological age measured in years for all human beings. The other type of ageing is not measured in the units of time but in terms of deterioration of biological functions. Both physiological and structural changes take place in the human body tissue. This progressive deterioration is called biological ageing. Biological and chronological ages are correlated, as the reduced physical ability to meet the requirement of an ageing human being is associated with the increase of chronological age (Adams and White, 2004).

There are four key mechanisms of cellular damage that contribute significantly towards the ageing process, i.e. the accumulation of non-functional aberrant proteins in the cytosol, mitochondrial functional changes, increased oxidative stress caused primarily by free oxygen radicals, and somatic mutations. These processes lead to DNA damage and repair mechanisms, resulting in abnormal cellular function, and contributing significantly towards both biological and chronological ageing (Kirkwood and Kowald, 1997). The accumulation of cellular damage is central to the pathogenesis of some of the most important causes of morbidity and mortality associated with ageing, including malignancy (accumulation of somatic mutations that lead to unrestricted growth potential and limitless proliferation), vascular endothelial dysfunction, or neurodegenerative diseases (Adams and White, 2004). Furthermore, environmental

factors, such as UV radiation, ionizing radiation, heavy metals, organic chemicals, food additives and smoking, have a pivotal role in the process of DNA damage (Rodriguez-Rodero et al., 2011). On the contrary, the anti-oxidant activities found in fresh fruit and vegetables, i.e. vitamin C and E and dietary derived minerals like selenium and manganese, are essential counterbalancing co-factors for antioxidant enzymes that slow down DNA damage and thus are capable of delaying the ageing process (Kenyon, 2010). An important aspect reported in many studies is the similarity between the ageing process and any other biological processes due to the regulation of specific genes and their associated pathways (Weinert and Timiras, 2003).

Recent studies have reported that many genes specially affecting insulin/IGF-1, TOR, JNK, protein translation and mitochondrial signalling, influence the lifespan of *C. elegans* (Wolff and Dillin, 2006; Braeckman and Vanfleteren, 2007; Tissenbaum, 2012). Earlier, Klass (1983), Friedman and Johnson (1988), and Kenyon et al. (1993) reported that it is possible to double the lifespan of a *C. elegans* by using chemical mutagenesis to introduce a targeted mutation in insulin/insulin- signalling. Though still controversial, genetic manipulation of genes in yeast, fruit flies, nematodes such as *C. elegans* and mice has suggested similar results (Sinclair and Guarente, 1997; Brown-Borg et al., 1996; Lin et al., 1998; Kenyon, 2010).

Stress-related genes have also been shown to influence the ageing process. Environmental stress and food availability could contribute to the control of the process of ageing exerted by stress related genes. Stress, nutrients, different environmental and physiological signals play a very important role in overall lifespan extension (Kenyon, 2010). Dietary restriction is the best known of those triggers that extend the lifespan of many species, from yeast to mice. *C. elegans* has been suggested as a good experimental module to study the genetics of lifespan in response to different diet restrictions (Greer and Brunet, 2009).

The genetics of *C. elegans* is well established, and its complete genome sequence provides an opportunity to disrupt gene function that might be involved in ageing. An important feature of *C. elegans* is its short life cycle in comparison to mammals, such as humans (Kenyon, 2010). Studying the effect of microbiota on ageing in the whole organism can reveal not only their conserved functions, but may also unravel novel contributions they provide in health and disease.

1.1.3 The Relationship between Ageing and Gut Microbes

Microbes live in a dynamic and complex relationship with their host within the mammalian gastrointestinal (GI) tract. Immediately after birth, microbiota of environmental, dietary, and maternal origin starts colonising the infant gut. For the maturation of the immune system of humans and for the development of mucosa in the intestine, these environmental microbes play an essential role from early infancy (Sekirov et al., 2010). Adult-type GI microbiota is relatively stable in the healthy adult until ageing occurs. However, the homeostasis of this adult-like microbiota starts responding to its pathophysiological processes. Only recently, research has focused on the influence of GI microbiota on longevity and health of human. The microbiota related phylogenetic and age correlated changes have been the basis of many recent studies to understand their importance in allergy and development of innate and cellular immune response. Recent advances in molecular technologies have contributed significantly for improving our understanding of the importance and nature of ageing by GI microbiota (Biagi et al., 2013).

1.1.4 Microbes Influence Ageing

As we age, there are subtle to substantial changes in our sense of smell, taste, and gastrointestinal motility, muscle functions that can also result in varied utilisation of

nutrition. These changes might exert influence on the physiology, absorption and metabolism of the host, ultimately contributing to the ageing (Orr and Chen, 2002; Woodmansey, 2007)

In the elderly, gastric degeneration has been reported to be responsible for the reduction in absorption of calcium, iron and vitamin B12 and results in an inability to release vitamin B12 from food or its binding proteins causing vitamin B12 deficiency (Biagi et al., 2012). It is evident from studies that GI microbiota has an important role to play in the health and disease of the host by regulating the digestion, absorption, and metabolic activities of the host and physiology. A careful study of GI microbiota may add to our knowledge for their further role in health and diseases and their contribution in the process of ageing.

1.2 Microbes and Inflammations

The term ‘inflamm-ageing’ was first coined in 2000 (Franceschi et al., 2000). It reflects the observations that elderly people exhibit reduced capability to cope with nutritional, physical, chemical, and antigenic stress, a phenomenon that is associated with a progressive concomitant increase in the level of numerous pro-inflammatory markers (Cevenini et al., 2013). These markers constitute a surrogate indication that a chronic inflammatory status is characteristic of the ageing process. Different types of cells in different tissues environments, such as gut microbiota, immune system, solid and hollow organs, muscle and adipose tissue, combine to produce local and secretory factors that interact with each other to curtail inflamm-ageing at both the systemic and local levels (Rehman, 2012). The importance to address the role of gut microbiota in longevity and ageing was suggested by many studies that conceptually linked these two topics (Cevenini et al., 2013). The immune system of the host is significantly affected

by the complex bacterial community, which exerts a systemic metabolic effect. This is because an evolutionarily adapted ecosystem represented by this complex bacterial community also helps to limit the potentially pathogenic bacteria and maintain the normal immune status.

Humans have a symbiotic relationship with their gut microbiota. Several transient and chronic clinical conditions, such as infections, allergy, diabetes and obesity, are subject to modifications by intestinal microbiota composition, which provides a direct means for microbiota to influence the wellbeing, ageing and the longevity (Biagi et al., 2010). Health status of the host is profoundly influenced by this homeostasis (Round et al., 2010). A state of 'constitutive low-grade physiological inflammation' mediated by microbiota sets the dynamics of cross talk between gut associated lymphoid tissue (GALT) (a part of the immune system) and gut microbiota. This allows the host to control and to tolerate the whole microbial complexity of gut microbiota while maintaining the normal immune response to various pathogen intruders. It is very important to preserve such symbiotic relationship between the host and the microbiota for the development of functional effective homeostasis and physiological mild inflammatory state. On many occasions, however, this homeostasis is lost in favour of microbiota, thus compromising the inflammatory status that leads to serious pathological conditions such as sepsis (Round et al., 2010; Rehman, 2012; Cevenini et al., 2013).

1.3 Zinc

There are many important microelements, such as magnesium, iron, copper and zinc, which play an important role in a plethora of biological processes (Bruinsma et al., 2008). In the human body, 2-3 grams of zinc are distributed in complexes with various

proteins (Haase and Rink, 2009). Zinc is an essential mineral plays a major role in regulating the structure and function of cell membranes and proteins, as well as catalytic enzyme activity in the host (King, 2011). At the cellular and biochemical level, zinc participates in multiple processes, with about 10% or 2800 proteins in the human genome identified as zinc dependent (Andreini et al., 2006), and is the only metal that appears in all enzyme classes. There are approximately 300 enzymes that depend upon zinc, and their function ranges from regulation of transcription (zinc finger motif containing transcription factors) (Haase and Rink, 2009) through antioxidant action (e.g. copper-zinc superoxide dismutase) to cell signalling that affects cell death and cell secretory processes. A critical role of zinc has been confirmed in biological processes including, but not limited to, transcription, translation, catalysis, cell division, development of innate and adaptive immunity, and nerve impulse transmission (Rink and Haase, 2007).

Considering the wide range of cellular roles that depend upon zinc, zinc must play a very important part in maintaining normal mammalian health. Zinc deficiency can lead to poor performance of zinc-dependent transcription factors, and other zinc-dependent enzymes, leading to a variety of abnormalities that manifest especially in reproductive, immune, nervous, integumentary and skeletal systems (Hambidge, 2000). More specifically, zinc deficiency can cause a decrease in the visual dysfunction, disturbed sense of smell and taste, hypogonadism, skin lesions, anorexia, poor wound healing, growth retardation and impaired immune function with susceptibility to infections (Haase and Rink, 2009). A chronic dietary zinc deficiency or malabsorption can result in several genetic diseases. *Acrodermatitis entropathica* is one of such conditions where mutation in the zinc transporters leads to metabolic disorder (Roh et al., 2012). On the other hand, excessive zinc is toxic (Roh et al., 2012) and may also cause dysfunction of proteins and the dislocation of metals to the different sites of affinity. In humans, it has

been reported that zinc toxicity is correlated to excess dietary intake, however it is rarely observed (Fosmire, 1990). Overall, an optimal level of zinc is necessary at the cellular level for the proliferation, differentiation, and normal homeostasis of cells (Haase and Rink, 2009).

Zinc has been shown to play a very important role in the function and development of the human immune system. Many recent studies have shown a correlation between zinc deficiency and age-related immunological dysfunction, as characterised by decreased immune responses and systemic chronic inflammation (Wong et al., 2013). Patients with low levels of zinc are also susceptible to infection and amplified inflammatory response (Besecker et al., 2011). In another study, a lowered level of chronic plasma zinc was observed with progressive ageing (Murr et al., 2012). The precise mechanisms linking zinc, ageing and inflammation remain, however, still not fully elucidated (Wong et al., 2013).

1.4 Zinc and its Absorption, Uptake and Regulation

In mammals, zinc is absorbed in the GI tract and then distributed through the circulating system (Krebs, 2000). Zinc homeostasis is maintained by critical processes that regulate zinc absorption, secretion, and excretion in the GI system, especially in the small intestine, liver and pancreas (Krebs, 2000). During digestion, zinc is released as a free ion from food, binds to endogenously secreted ligands and is transported into enterocytes in the duodenum and jejunum (Roohani et al., 2013). Zinc is a hydrophilic ion, and it cannot be transported across membranes by passive diffusion. Therefore, the integral membrane proteins are required to facilitate zinc ions movement across the cytoplasmic membrane into the portal circulation to be delivered to the other tissues. There are several transport mechanisms, which are needed for the entrance and the exit

of zinc in both eukaryotes as well as prokaryote cells (Eide, 2006; Roohani et al., 2013).

1.4.1 Zinc Transporters in Mammals

The SLC30A (Solute linked carrier 30A) and SLC39A (Solute linked carrier 39A are two significant families of mammalian zinc transporters (Palmiter and Huang, 2004). The SLC30A proteins are also known as CDF/ZnTs (Cation Diffusion Facilitator– Zn Transporters). Their main function is associated with compartmentalisation and efflux of zinc. These transporters are needed to reduce intracellular zinc levels by transporting zinc from the cytoplasm to the lumen of organelles or across the plasma membrane (Eide, 2006; Palmiter and Huang, 2004; Lichten and Cousins, 2009). The SLC39A is a member of ZIPs family (Zrt-, Irt-like Protein) which was named after the yeast Zrt1 (zinc-regulated transporter) protein and the Arabidopsis Irt1 (iron regulated-transporter) protein. The primary function of this family is to increase intracellular zinc levels by either transporting the metal from extracellular space or organelle lumen into the cytoplasm (Lichten and Cousins, 2009; Taylor and Nicholson 2003). In mice and in humans there are 14 members of the ZIP family identified thus far which participate in the uptake of zinc (Eide, 2006; Guerinot, 2000). There are eight trans-membrane domains of these protein families and the spanning domain IV consists of preserved serine, glycine and histidine residues that are present in an amphipathic α-helix. The ZIP proteins are also involved in the initial development of the zebra fish embryo and in the process of homeostasis of zinc (Yamashita et al., 2004). Based on its structural homology, the ZIP family of proteins is further subdivided into four sub- families (Taylor and Nicholson, 2003). The members of subfamily LIV-1 have the function in the transportation of zinc in transfected cells (Wang et al., 2004). The members of subfamily II have a conserved amino acid sequence, and it is conserved in the domain IV. The amino acid sequence homology

between human and mouse proteins ranges from 83% to 93%. Though both ZIP1 and ZIP3 are expressed in the embryonic tissues, the gene expression of ZIPI is higher than ZIP3 (Dufner-Beattie et al., 2005). The abundance of *ZIP4* mRNA in the mouse is inversely proportional to the zinc levels (Dufner-Beattie et al., 2003), whereas the sub-cellular localization of ZIP1, ZIP3, ZIP4, and ZIP5 are dependent on zinc levels. Under zinc limiting conditions, mice lacking ZIP1, ZIP2 or ZIP3 show abnormal embryogenesis. Though over the years much information have accumulated for ZIP family protein, we understand little about the regulation, expression, and functions at cellular level in-vivo (Dufner-Beattie et al., 2005).

ZnT- 1, 2, 3 and, 4 play crucial roles in zinc influx and efflux across the plasma and intracellular membranes. ZnT1 is expressed ubiquitously and is localised predominantly in the intestinal membrane (Palmiter and Huang, 2004). Studies in ZnT-1 knockout or transgenic mice have shown that ZnT-1 has a role in an early developmental phase (Andrews et al., 2004). Similarly, studies with mammalian ZnT-2 mutants have suggested poor absorption of nutritional zinc in early neonatal life (Palmiter et al., 1996). Deficiencies of ZnT-3 and ZnT-4 have been implicated in poor neuronal functions (Cole et al., 1999) and truncated zinc availability in secretory vesicles of mammary glands, respectively (Erway and Grider, 1984; Huang et al., 1997). Male mice lacking ZnT-5 were phenotypically characterised by reduced osteoblast formation, cardiac defects, lean body, and muscle weakness (Inoue et al., 2002). ZnT-6 is mainly expressed in brain, liver, and small intestine, and its intracellular localisation is in the trans-Golgi network (Huang et al., 2002). ZnT-7 mRNA has been detected in a wide variety of tissues including liver, small intestine, and spleen, and in terms of intracellular localisation ZnT-7 protein is found in the Golgi apparatus. ZnT-8 is mainly expressed in pancreatic β cells, localised to insulin-containing granules, and is involved in zinc accumulation and efficient insulin storage (Chimienti et al., 2005). The

expression patterns and localisation of the majority of the predicted mammalian CDF proteins and mutant analyses have defined functional roles for ZnT-1, ZnT-3, ZnT-4, and ZnT-5 (Roh et al., 2013). Nevertheless, the mechanisms that regulate zinc absorption, usage, storage, excretion and handling of excess zinc by the animals are still elusive, and warrant more studies.

1.4.2 Zinc Transporters in *C. elegans*

The *C. elegans* nematode is a multicellular organism that has relatively simple anatomy and is ideal for molecular and genetic analysis. *C. elegans* are small, free-living organisms, with a large brood size, short life cycle, and fully-sequenced genome (Brenner, 1974). Adult *C. elegans* hermaphrodites have 959 somatic nuclei resulting from an almost invariant developmental program for which the cell lineage has been fully described. The worm's anatomy has been extensively described using electron microscopy and can be directly visualised using differential interference contrast imaging since the worm is translucent. Methods for conducting forward genetic screens, creating transgenic animals, and defining protein localisation are readily available (http://www.wormbook.org).

Ease of culturing and powerful genetics has encouraged the development of *C. elegans* as a model system to study human biology. Indeed, many central pathways involved in human development and disease are conserved in *C. elegans*, for example apoptosis (Leung et al., 2008). Together, these characteristics make *C. elegans* an ideal choice for the study of zinc metabolism (Bruinsma et al., 2002; Davis et al., 2009a; Roh et al., 2012; Roh et al., 2013). The *C. elegans* is a better model to study zinc metabolism than yeast or rodents, because yeast lack the complexities of a multicellular animal and the genetic analysis of rodents is still challenging.

Relatively little is known about the regulation of zinc metabolism in *C. elegans.* Metallothioneins (MTs) exhibit strong affinity for metals and have been shown to contribute significantly to metal detoxification and homeostasis in most organisms, including *C. elegans* (Leszczyszyn et al., 2011). Two inducible isoforms CeMT1 and CeMT2 (mtl-1 and mtl-2) are present in the *C. elegans* gut and are involved in the protection from zinc and cadmium toxicity, respectively, as deletion of CeMT-1 gene results in significant accumulation of zinc, whereas deletion of CeMT-2 resulted in higher accumulation of cadmium. These interesting findings suggest strongly that zinc homeostasis is tightly regulated in *C. elegans*. A complete sequence of *C. elegans* genome showed a family of 14 genes that encode CDF (Cation Diffusion Facilitator) protein. Only *cdf- 1* and *sur-7* genes have been identified, during mutational studies, to be involved in progressive survival during high dietary zinc experiments. These studies showed that these genes play an important role for zinc tolerance (Bruinsma et al., 2002; Yoder et al., 2004). Further, their findings suggested an unexpected connection between zinc metabolism and signal transduction suggesting that cytosolic zinc concentrations modulate the activity of the Ras/MapKinase signalling pathway (Bruinsma et al., 2002; Yoder et al., 2004). Consistent with a role for CDF-1 in zinc metabolism, *cdf-1* mutations caused hypersensitivity to supplemental dietary zinc (Bruinsma et al., 2002; Bruinsma et al., 2008). ZnT-1 is the mammalian homolog to CDF-1 as ZnT-1 can functionally replace CDF-1 in transgenic worms.

Since ZIP and CDF families of metallothioneins are present in *C. elegans*, the mechanisms of zinc metabolism could be similar as compared to the other animals. It has been reported that in *C. elegans* fed with a zinc diet of 30 μM to 1 mM zinc localises in the vesicles of intestinal cells (Davis et al., 2009a). Such information warrants further investigations to characterise zinc storage site and its mobilisation during active transportation.

Zinc-specific fluorescent dyes are usually used to achieve this objective. Using such methodology it has been shown that zinc storage is CDF-2 zinc transporter dependent, and that zinc is stored in the intestinal bilobed morphology lysosomal organelles. These characteristics play an important role in the mobilisation and detoxification of the animal in the physiological setting (Roh et al., 2012).

1.4.3 Zinc Transporter in *E. coli*

Zinc, a vital element, is necessary for the normal physiological functions of organisms, but at high concentration can be toxic. There are various mechanisms to regulate cellular zinc homeostasis in prokaryotes. Bacterial cells may use zinc to achieve the critical balance between its supply and demand and to avoid zinc toxicity. The bacterial cell use a very efficient system of high and low affinity binding proteins to regulate, store and keep optimum concentration of zinc to maintain their normal function (Hantke, 2005). Zinc storage proteins, however, are not very common in bacteria (Cerasi et al., 2013). Cyanobacteria are the exception, in which zinc is detoxified and stored by the metallothionein SmtA (Phan et al., 2004). Zinc concentration balance in *E. coli* is regulated with the help of CDF *ZitB* and the P-type ATPase ZntA (Grass et al., 2001). ZntR (Zn-responsive Mer-like transcriptional regulator) regulates the synthesis of ZntA by binding to zinc, thus allowing the excess metal efflux from the cell (Pruteanu et al., 2007). Uptake of zinc is ensured by transporters characterised by different affinity for the metal (Hantke, 2005). When bacteria grow in environments with moderate zinc concentration, metal uptake is carried out by the low affinity permease ZupT, a member of the ZIP family of transporters. In contrast, when bacteria grow in very low zinc concentration environments, characterised by very low zinc availability, zinc import is ensured by the high affinity zinc transporter *znuABC* (Yatsunyk et al., 2008), synthesis of which is tightly controlled

by the binding of zinc to the promoter of the *zur* gene (Patzer and Hantke, 2000).

ZnuABC

Zinc-specific uptake, first identified in *Escherichia coli,* is mediated by the ATP- binding cassette znuABC transporter family. ZnuABC is expressed under low external concentration of zinc and contains three constituents: i) the ZnuA protein, the soluble periplasmic metallochaperone that captures zinc *via* its cellular domain and then delivers it to the transmembrane element of the transporter (Ammendola et al., 2007); ii) ZnuB, an integral membrane protein which mediates transportation of zinc through the cytoplasmic membrane; and iii) ZnuC ATPase component which delivers the energy necessary for ion transport through the inner membrane (Yatsunyk and Rosenzweig, 2007; Cerasi et al., 2013).

Studies using biochemical and genetic approaches have revealed that in cells containing sufficient concentrations of zinc *znuABC* expression is blocked by Zur (zinc uptake regulator), a metalloregulatory DNA-binding protein with femtomolar sensitivity to free intracellular zinc (Outten and O'Halloran, 2001).

Studies conducted in different bacterial species recognised *znuABC* as necessary to allow efficient bacterial growth in zinc deficient media and to ensure bacterial virulence. It has been shown that in an infected host, zinc presence is very limited and several bacteria strictly rely on *znuABC* to compete with their host for zinc binding (Ammendola et al., 2007; Sabri et al., 2009; Chen and Morse, 2001).

1.5 Competition for Zinc between Pathogen and Host

The competition for iron between the bacteria and their host has been well documented, whereas the importance of zinc interaction between host and bacteria has been

investigated only recently. It has been shown *in vitro* that deletion of the *znuABC* genes not only decreased bacterial ability to grow in metal deficient environments, but also severely affected their pathogenicity (Hood et al., 2012). Several studies (Patzer and Hantke, 1998; Chen and Morse, 2001; Campoy et al., 2002; Garrido et al., 2003; Gielda and DiRita, 2012) proposed that active bacterial infections with species such as *Salmonella enterica*serovar, *Typhimurium, Campylobacter jejuni*, *Neisseria gonorrhoeae, Pasteurella multocida, Brucella abortus, Haemophilus ducreyi, and Haemophilus influenzae* are dependent on *znuABC* and on amount of zinc availability. These hypotheses have not been, however, fully proven and there are contradicting reports suggesting the role of *znuABC* in bacterial pathogenicity needs to be further examined (Ammendola et al., 2007).

1.6 Zinc Toxicity in *C. elegans*

As described earlier (in section 1.4.2) *C. elegans* is a good working system for studying metal biology, and it can be used to analyse zinc signalling, metabolism and metal toxicity (Bruinsma et al., 2002; Bruinsma et al., 2008; Davis et al., 2009a; Murphy et al., 2011). There are well-documented studies in *C. elegans* investigating how metal toxicity affects lifespan, including mortality and reproduction (Jones and Candido, 1999; Boyd et al., 2003). Acute toxicity of metals has also been reported on the behavioural level, including worm's locomotive behaviour, head thrashing, body bending, and other basic behaviours (Dhawan et al., 1999).

A genetic screening was performed for those mutants of *C. elegans* which were resistant for the toxicity of zinc (Murphy et al., 2011). These studies revealed that the mutation of the gene of histidine ammonia lyase (*haly*-1) in *C. elegans* promoted resistance to high levels of zinc. *C. elegans haly*-1 is an enzyme which transforms

histidine to urocanic acid. Animals harbouring the mutation in *haly*-1 had increased levels of histidine, supporting the notion that *haly*-1 is necessary for the catabolism of histidine. The results have suggested that when the levels of histidine are increased, the toxicity of zinc can decrease *via* by the chelation of zinc. The study also indicated that the tolerance levels of zinc are increased by dietary histidine.

Metal chelators or immobilisers (such as desferrioxamine B, 3-hydroxy-1, 2-dimethylpyridin-4-one (DMHP), and acetohydroxamic acid (AHA), iron-specific chelators; cyclam, cysteine, and histidine, zinc and copper chelators; and calcium EDTA (CaEDTA) are reported as a potential protector against metal contamination in the environment (Harrington et al., 2012). The study also observed that with the application of metal chelators there was minimal toxicity at high metal concentration, resulting in a minor effect on the worms' growth. The results of these studies suggested that chelating agents are most suitable for protecting *C. elegans* against environmental metal toxicity.

1.7 *C. elegans*, Gut Microbes, Ageing and Zinc

As described earlier, gut microbiota contributes not only in the host metabolic pathways, but is also significantly involved in metabolic and inflammatory disorders such as inflammatory bowel disease, diabetes, obesity, liver disease, and cancer (Nicholson et al., 2012). Recently, researchers have been focusing on the influence of microbiota on the ageing process. Considering the complexity and the heterogeneous nature of the mammalian gut microbiota, however, it is experimentally challenging to define their exact role and the interaction between microbes and their host. For this very reason *C. elegans* proved to be a very informative and simple model for gene

manipulation experiments to obtain meaningful data for ageing research (Cabreiro et al., 2013).

C. elegans is ideal for such studies, since under standard culture conditions only a single microbe (*E. coli,* a resident human gut bacterium) is present as a food source (Brenner, 1974). *C. elegans* has been used to study the effect of manipulated nutrients to slow development, increase lifespan and decrease fecundity (Lenaerts et al., 2008).

Virk et al. (2012) observed that the rate of ageing in *C. elegans* could be slowed by mutation in the *aroD* gene, which is necessary for synthesis of aromatic compounds in bacteria. Adding the aromatic component paraaminobenzoic acid (PABA) to the *C. elegans* nutrient growth media (NGM) reversed the worms' lifespan increase, suggesting decreasing accessibility of a folate precursor, para-aminobenzoic acid, could increase the worms lifespan. Consistent with the result, a dose-dependent increase in the lifespan of *C. elegans* results from inhibiting folate synthesis by sulfamethoxazole (Virk et al., 2012).

Because *aroD* was found accidentally, a random unbiased genetic screen was performed to see if there were any more gene deletions that increased *C. elegans* lifespan. From 1041 genes, 9 genes were found to increase *C. elegans* lifespan. One of the genes was *znuB* (Jia et al, *unpublished data*) that forms an important part of the high affinity *znuABC* zinc ABC transporters needed to transport zinc through the cell membrane when the external zinc concentration is low (See section 1.4.3). These results suggested that decreasing zinc uptake could increase worms lifespan. Zinc is regarded as an essential element; however, according to (Wang et al., 2007) zinc exposure to *C. elegans* results in ageing of worms and could effect on shortening their lifespan.

Recent studies have revealed an important role of *znuABC* protein during host-bacteria interaction (See section 1.5) (Ammendola et al., 2007; Gielda and DiRita,

2012). We hypothesise that the high affinity zinc uptake system plays an important role in bacteria and might also influence the lifespan of *C. elegans*. In the present study we investigated the host-microbiota interaction using a worm model.

CHAPTER 2: MATERIALS AND METHODS

2.1 *Caenorhabditis elegans* and *Escherichia coli* Strains

In studies of *C. elegans* lifespan and for metal analysis I used a SS104 *glp-4* (*bn*2) strain. This strain is temperature sensitive with regards to its fecundity (Beanan and Strome, 1992). It is fertile at 15°C but infertile at 25°C, which makes lifespan experiments at 25°C (infertile worms) easier. The wild type N2 strain was used for the fecundity analysis (Virk et al., 2012).

I used bacterial strains from the Keio collection (Baba et al., 2006) (Table 2.1). Some of those strains transformed with plasmids in our lab (Jia et al, *unpublished*) were also used. The OP50 bacteria were used for worm maintenance (Brenner, 1974).

Strain	Strain name	Genotype	Source	Antibiotic Resistance
OP50	OP50	*Ura*	(Brenner, 1974)	-
WT	BW25113	F-, *Δ (araD-araB) 567*, *ΔlacZ4787* (:rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	(Baba et al., 2006)	Kanamycin
znuA	JW5831	BW25113, *ΔznuA782::kan*,	(Baba et al., 2006)	Kanamycin
znuB	JW1848	BW25113$^-$, *ΔznuB784::kan*,	(Baba et al., 2006)	Kanamycin
znuC	EJW1847	BW25113$^-$, *ΔznuC783::kan*,	(Baba et al., 2006)	Kanamycin
WT: vector	ΔWT::WT	BW25113, *ΔWT- pMM67EB*	Jia et al, *Unpublished*	Carbenicillin
znuB: vector	SKV1859	BW25113, *ΔznuB- pMM67EB* (JW1848)	Jia et al, *Unpublished*	Carbenicillin
znuB: Gene Complementation	SKV1859	BW25113, Δ*znuB*- (JW1848) *pMM67EB::znuB*	*Jia et al, Unpublished*	Carbenicillin

Table 2.1: The strains of *E. coli* used in the current study.

2.2 Chemicals

Peptone (Sigma P6713), High Purity Agar (Sigma 05038), NaCl (S7653), Cholesterol (Sigma C8667), Ethanol (Sigma E7023), $MgSO_4$ (Sigma M1880), $CaCl_2$ (Sigma C3881), KH_2PO_4, (Sigma P0662), $ZnSO_4.7H_2O$ (BDH 10299), Kanamycin (Sigma K0126), Carbenicillin (Sigma C1389), $Na_2HPO_4.2H_2O$ (Sigma S9763), NaOCl (Sigma 425044), and NaOH (Sigma S7653).

2.3 Media and Buffer Preparation

NGM medium: NGM nematode growth media (Brenner, 1974) was prepared by dissolving 2.25 g peptone, 18 g high purity agar[1], and 2.75 g NaCl in 900 ml distilled water. After autoclaving for 15 minutes at 121°C, 0.9 ml of 5 mg/ml cholesterol in ethanol, 0.9 ml 1 M $MgSO_4$, 0.9 ml 1 M $CaCl_2$ and 22.5 ml 1 M KH_2PO_4, buffer pH 6.0 were added in that order. 15 ml of media was poured into petri dishes (6 cm). After agar solidification, the plates were stored at 4°C, for long-time storage.

When needed, $ZnSO_4.7H_2O$ was added at a final concentration of 1 μM, 10 μM, and 100 μM in NGM. Also when needed, antibiotics were added into the media, Kanamycin; added freshly at a final concentration of 50 μg/ml, or Carbenicillin; added freshly at a final concentration of 100 μg/ml.)

LB medium: Luria Broth is a highly referenced microbial growth medium that contains peptides, amino acids, water-soluble vitamins, and carbohydrates in a low-salt formulation used for the culture of *E. coli*. This media consists of 10 g tryptone, 5 g

[1] In our previous studies, we found variation in lifespan on the worms grown on standard agar and therefore high purity agar was used rather than standard agar.

yeast extract and 10 g NaCl in 1 litre of distilled water, autoclaved for 15 minutes at 121°C.

Preparation of bacterial culture: *E. coli* is the food source for the worms. To prepare culture that will be available when needed 30 ml of LB was pipetted into sterile 50 ml falcon tube with the appropriated antibiotic. A disposable inoculating loop was used to inoculate the LB with appropriate bacteria from a frozen stock prepared previously. Once inoculated tubes were placed at 37°C on an orbital shaker set on 80 rpm and allowed to grow overnight. Cultures were then used for seeding NGM plates or stored at 4°C until needed (for good practice I never freeze-thaw frozen stock more than 2 times and always prepared fresh culture for seeding plates).

M9 buffer: M9 is a solution that used to keep the osmotic value in balance, so the adult worms can be maintained. To make M9 buffer, 5.8 g $Na_2HPO_4.2H_2O$, 3.9 g KH_2PO_4, 0.25 g $MgSO_4$, and 5 g NaCl were dissolved in 1L distilled H_2O, autoclaved for 15 minutes at 121°C, and kept sterile until needed.

2.4 Preparation of *C. elegans* for Experiments

Protocol of *C. elegans* preparation was consistent with previously published guidelines, and used for all the experiments conducted during this study (Figure 2.1).

Eggs were prepared by treating adult hermaphrodites with alkaline bleach (7:8: NaOCl: 4 M NaOH) to remove the cuticle and any microbes attached to the worms. The eggs were washed with M9 buffer 3 times, and then placed and pre-seeded on NGM plates with 100 µL OP50 bacteria (Weinkove et al., 2006). Animals were then left to grow at 15°C until adulthood, and then transferred onto pre-seeded fresh kanamycin-supplemented NGM plates with appropriate bacteria ± relevant $ZnSO_4.7H_2O$. Hermaphrodites were left to lay eggs overnight, and then removed. Progeny were

allowed to grow and develop at 15°C until L3/L4. Then the plates were transferred to 25°C. From this point worms were used for 3 different studies, as summarised in Diagram 1, and will be described under each of the following experiment protocols in detail.

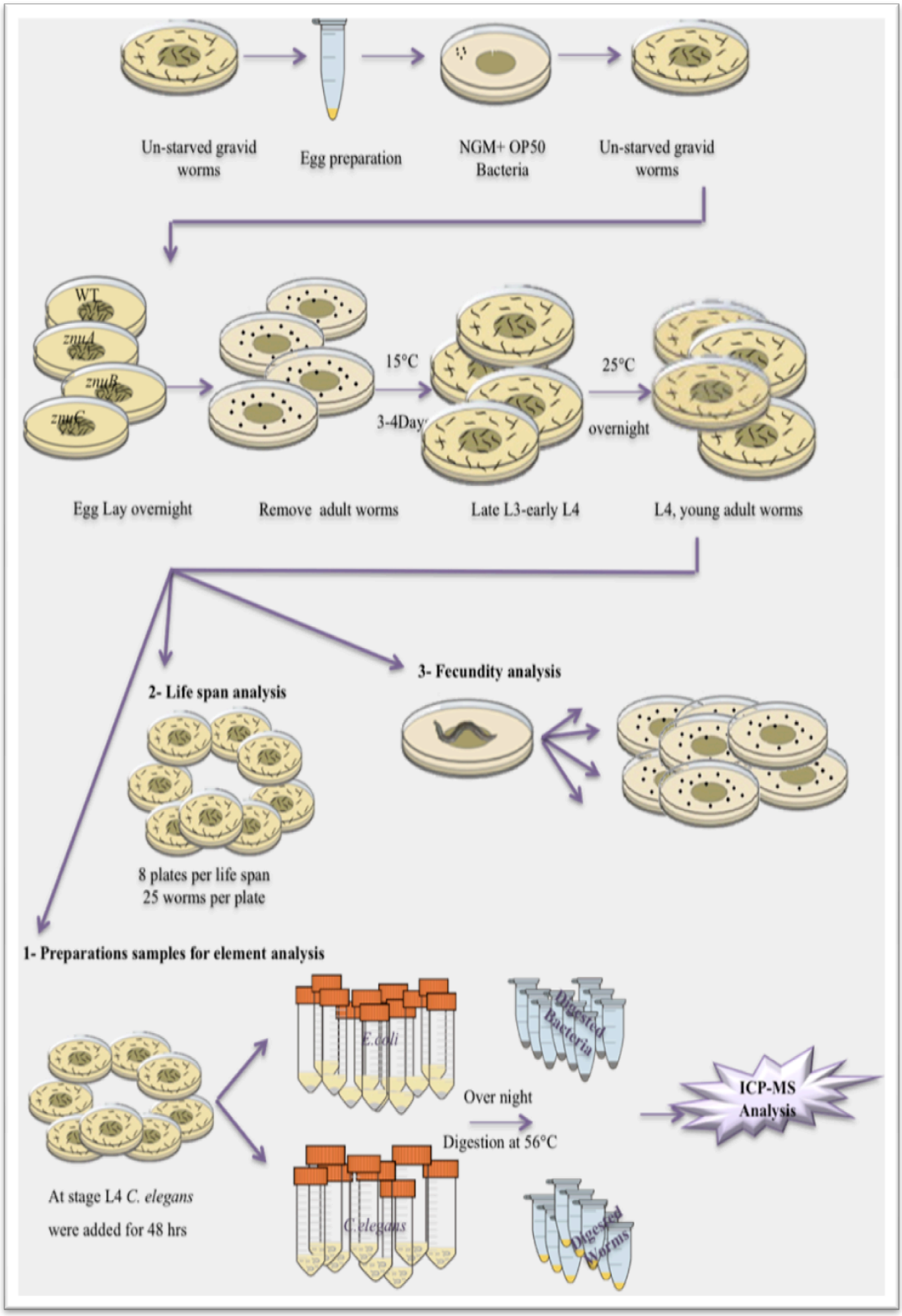

Figure 2.1: Schematic showing *C. elegans* preparation for the usage in 3 types of experiment: 1-Elemental, 2-Lifespan, and 3-Fecundity analysis.

2.5 Elemental analysis

2.5.1 Materials and chemicals used

Name	Catalogue Number
Metal free tips 200 µl	VWR 732-0643
Metal free tips 1000 µl	VWR 732-0647
Metal free 15 ml tubes	VWR 89049-170
Metal free 50 ml tubes	VWR 89049-174
65% Nitric acid	Merck 2261141213
EDTA (Ethylenediaminetetraacetic acid)	Sigma E6758
$Na_3C_6H_5O_7$ (Tri-sodium citrate)	Sigma S1804
Silver (I) single element solution 1,000 µg/g matrix of 1M nitric acid in plastic container atomic absorption spectroscopy Standard Metal Solution	Fisher10568050
Zinc single element solution 1000µg/g matrix of 1M nitric acid in plastic container atomic absorption spectroscopy Standard Metal Solution	Fisher 10179031
Iron single element solution 1000 µg/g matrix of 1M nitric acid in plastic container atomic absorption spectroscopy Standard Metal Solution	Fisher 10533142
Manganese single element solution 1000 µg/g matrix of 1M nitric acid in plastic container atomic absorption spectroscopy Standard Metal Solution	Fisher 10382562
Copper single element solution 1000 µg/g matrix of 1M nitric acid in plastic container atomic absorption spectroscopy Standard Metal Solution	Fisher10743173
Cobalt single element solution 10000 µg/g matrix of 1M nitric acid in plastic container ICP (OES or MS) Primar	Fisher 10356090
Freeze dryer	Christ Alpha 1-4 LSC
ICP-MS Thermo Scientific Inductively Coupled Plasma-Mass Spectrometry.	Thermo Scientific X-Series II

Table 2.2: Materials, chemical reagents, and equipment used for element analysis[2].

[2] Being particularly carful in sample preparation for ICP-MS analysis was necessary in this study, because the results can be influenced by contaminating metals from the environment (Hsiung et al., 1997; Davis et al., 2009a). To remove all potential contamination with metals, the bottles were washed with 4% nitric acid and then rinsed

2.5.2 *C. elegans* and *E. coli* Preparation for Element Analysis

To get large quantities of SS104 worms, we used large plates (9 cm in diameter) with double peptone NGM medium, pre-seeded with 200 µl appropriate bacteria. Worms were prepared as described in 2.4. After incubating L4/ young adult worms overnight at 25°C, we rinsed the warms off the plates using ddH_2O and placed them onto new seeded plates, leaving them at 25°C for 2 days with appropriate bacteria ± relevant concentration of $ZnSO_4.7H_2O$. After this period of incubation, we washed the worms and the bacteria off the plates, using ddH_2O, collected them in 50 ml tubes, and then spun at 20 g for 10 minutes in order to collect the worms. Subsequently, we transferred the supernatant into fresh 50 ml tubes, and centrifuged it at 2500 g for 15 minutes to collect the bacteria. The worm pellets from the first centrifugation were re-suspended in ddH_2O and washed 3-4 times with ddH_2O until supernatant was clear, indicating that worms were free of most of the bacteria. The clean worms were pelleted by centrifugation at 100 g for 10 min.

The worm and bacterial pellets were weighed, excess supernatant (H_2O) were removed and kept frozen at -80°C. Later, samples were re-suspended in SSW buffer to remove surface-bound zinc, (Simm et al., 2007) (1 mM EDTA, 1 mM KH_2PO_4, 20 mM $Na_3C_6H_5O_7$ (Tri-sodium citrate), 1 mM $CaCl_2$, 5mM $MgSO_4$, and 1 mM NaCl) and then spun at 21,000 g. Samples were weighed again, taking as much liquid as I could (to use numbers in data analysis later on), and then 1 ml of 65% Nitric Acid (HNO_3) was added

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thoroughly with double distilled H_2O (ddH_2O) before use. The bottles were not autoclaved to prevent any additional metal contamination from such sterilisation process. Metal free tips and tubes were used (Graham et al., 2009).

to the pellet, for each sample digested overnight at 55ºC. Samples were spun at 21,000 g to collect the supernatant. To make sure that the acid had digested worms and bacteria completely, tubes were visually inspected for sign of pellet. The clear supernatant diluted 1:10 in internal standard solution that consists of 2.5% HNO_3 and 10 ppb $AgNO_3$. The standard curve was made by known dilutions of mixed standard solutions for 5 metals (Zn, Mn, Fe, and Cu 1000 ppm of each and Co 10,000 ppm) dissolved in the internal standard solution. Samples and control in internal standard solution was introduced into X-Series II ICP-MS[3] Thermo Scientific Inductively Coupled Plasma-Mass Spectrometry using PlasmaLab program to run samples through (Graham et al., 2009; Davis et al., 2009a; Dainty et al., 2010).

The zinc concentration in the samples in ppm was calculated using the following formula with assumption of elemental composition was constant:

[(Sample reading for ^{66}Zn) ÷ Sample weight]

For molar concentrations the following formula was used:

(Reading (^{66}Zn) / MW for ^{66}Zn) / sample weight

2.5.3 Element Analysis in Media

Zinc concentration in media analysed in 2 different ways:

A) 1 ml kanamycin supplemented NGM ± $ZnSO_4$ in 3 different concentrations were freeze-dried and digested in 65% nitric acid at 55ºC overnight. These samples were

[3] Every time we run samples though the ICP-MS, we calibrated the machine by using freshly prepared known standard solution, diluted in internal standard solution (2.5% HNO_3 and 10 ppb $AgNO_3$), for each sample and control, to adjust for the matrix effect in the instrument (Hsiung et al., 1997; Davis et al., 2009a).

introduced into the ICP-MS[3] and analysed as described above in 2.5.2.

B) 1ml kanamycin supplemented NGM ± $ZnSO_4$, 1 ml H_2O± $ZnSO_4$, 1 ml kanamycin supplemented NGM without agar media ± $ZnSO_4$, in 3 different concentrations, were heated at 55ºC for 2 days in 15 ml metal free tubes dried 100 µl LB media. The residues were digested in 65% nitric acid at 55ºC overnight and analysed by ICP-MS[3] as described above in 2.5.2.

2.6 Lifespan Analysis

SS104 worms were prepared as described in 2.4. After growing worms in the appropriate bacteria at 25°C for 3 days, 25 adult worms were transferred to individual plates, with 8 plates for each condition (Virk et al., 2012). Different conditions include exposure to different strains of bacteria and relevant of supplemented zinc.

Animals on appropriate bacteria were kept at 25°C for 7 days. They were then transferred to fresh plates with the same bacteria after 7 and 14 days and scored for survival every 2 or 3 days. Lifespan data were analysed by JMP statistical software (SAS institute Inc, Cary, NC, USA). Determination of relevant, statistical significance was made by the Log-Rank and Wilcoxon tests of fitting to the Kaplan-Meier survival model (Virk et al., 2012).

2.7 Fecundity analysis

Fecundity assay was performed with N2 worms kept on different kinds of bacteria (WT*, znuA, znuB, and znuC)* with and without 10 µM zinc on kanamycin supplemented NGM plates. NGM media was prepared as described previously, and dispensed in (3.5 cm) small plate. N2 worms were cultured from eggs at 15°C on

appropriate bacteria (WT*, znuA, znuB, and znuC)* on NGM plates ±10 μM zinc until L4. The worms were then shifted to 25°C, from the beginning of adulthood individually. Animals were transferred to fresh plates every 24 hours until egg laying stopped. The progeny from each plate was allowed to develop for 3 days in 25°C then counted (Virk et al., 2012).

Another fecundity assay was performed with N2 worms kept on different kinds of bacteria (WT*, znuB, and* gene complemented *znuB)* on carbenicillin supplemented NGM plates rather than kanamycin supplemented NGM plates.

2.8 Imaging Developmental Rate

Worms grown on *znuA, znuB*, and *znuC* bacteria were imaged every other day from day 5, till day 13 of their life cycle and compared with worms fed with WT bacteria using a Leica, M165FC microscope, a Leica DFC420 C camera, and the Leica Application Suite software. Images were taken with the same software setting and camera magnification.

CHAPTER 3: RESULTS

3.1 Zinc Concentration Measurement

3.1.1 Measurement of Zinc Concentration in *E. coli* and *C. elegans*

The present study was undertaken to understand the role of *znuA, znuB*, and *znuC* mutations in *E coli* on the concentration of zinc both in *E. coli* and worms fed with them in culture. A number of experiments were performed to measure the zinc concentration in the three mutant strains and as well as the worms fed with these strains. The zinc concentrations were also compared to the concentration of zinc in WT bacteria and worms fed with those bacteria. The experiments were performed three times independently on different days keeping similar experimental conditions. The data presented here is an average of data from these three independent experiments.

As was described in section 2.5.2, the zinc concentration in the samples was calculated using the following formula with the assumption that elemental composition was constant

^{66}Zn = [(Sample reading for ^{66}Zn) ÷ Sample weight]

As described earlier (Murphy et al., 2011) the *C. elegans* weight was determined by freeze-drying technique i.e. pre-weighed tubes containing worms and reweighing to obtain the dry weight. In another study by Page et al. (2012) the heat drying pre-weighed tubes containing worms and reweighing to obtain the dry weight were used. In the current study I weighed my samples by using ultracentrifuge, spinning pre-weighed frozen samples first at 21,000 g and weighed tubes immediately before digestion with nitric acid to extract maximum zinc.

The experiments revealed interesting association between the genotype of the bacteria and the zinc content in both bacteria and worms fed with them. Wild-type (WT) bacteria had zinc content higher than the 3 mutant strains (*znuA, znuB*, and *znuC*), with mutant bacteria reaching ≤45% the zinc content of the WT strain (Figure 3.1). Worms fed with WT strain reached zinc concentration that was 42% of zinc concentration in the bacterial strain they were fed with, whereas worms fed with the mutant strains *znuA*, *znuB* and *znuC* showed zinc concentration which was 72%, 74%, and 66% of zinc concentration in the respective bacterial strains. Zinc concentration in worms fed with mutant strains *znuA*, *znuB* and *znuC* was 78%, 77%, and 71%, respectively, compared to zinc concentration detected in worms fed with WT bacteria (Figure 3.1).

These results support the hypothesis that *znuABC* is involved in taking up zinc when the external zinc concentration is low (Ammendola et al., 2007). Therefore, increasing the external zinc concentration should reverse the effects of *znuABC* mutants. To test this hypothesis, experiments were performed to see if adding external different concentrations zinc to the NGM media could increase zinc concentration in the mutant bacteria as well as worms fed with them. Zinc concentrations in mutants and worms fed with them were compared to the concentration in WT bacteria and worms fed with them under same conditions.

As shown in the Figure 3.2 and Table 3.1, addition of 1 μM of zinc in NGM significantly increases zinc concentration in the wild type and the 3 mutant bacteria ($p<0.05$) as compared to controls. Interestingly, no significant difference was observed at higher concentrations of zinc, i.e. 1 μM and 10 μM ($p>0.05$).

Adding 100 μM external zinc in NGM showed statistically significant rise in the wild type and the 3 mutant bacteria ($p<0.001$). Worms fed with the 4 bacterial strains after 1 μM and 10 μM external zinc supplementation in NGM showed a steady rise in

the zinc concentration compared to worms fed with the 4 bacterial strains that were cultured in normal (non-supplemented) NGM media, although statistical significance was not reached. Worms fed with 4 strains in the presence of 100 μM external zinc in NGM showed double zinc concentration compared to the worms fed with 4 strains in normal NGM media ($p<0.05$). However, the zinc concentration in the worms fed with 4 strains in the presence of 100 μM external zinc still was lower than the zinc in the 4 bacterial strains. These results are consistent with several previous studies showing that *C. elegans* cultured with high dietary zinc shows elevated level of total zinc and that excess zinc can be stored in worms (Davis et al., 2009a; Roh et al., 2012).

3.1.2 Measurement of Zinc Concentration in the Media

In order to investigate where the bacteria and worms get their zinc from, zinc concentration was measured in both NGM and in LB media. Two different methods were used to quantify zinc concentration in NGM media. First, zinc concentration was measured by freeze-drying 1 ml of NGM media with and without different zinc supplements. Zinc content data obtained with such analysis were inconsistent with the known zinc supplementation added (Figure 3.3A, table 3.2). To understand this variability, I measured zinc using a different method, whereby zinc concentration in NGM was compared with NGM without agar media, and H_2O, with and without different zinc concentration supplements. To eliminate the possibility that the vacuum used in freeze-drying was causing sample loss, I dried samples by heating in an incubator. As shown in Figure 3.3 B and Table 3.2 the concentration of zinc in the NGM with agar media were inconsistent, compared to the zinc concentration in the NGM without agar media and in the H_2O.

These results are suggestive of the fact that a matrix effect can be produced by

the presence of agar in the NGM media, making it impossible to achieve consistent measurements of zinc content using ICP-MS. According to manufacturers, the element analysis in agar was done by the ICP-OES and zinc is included among the standards. They report calcium ≤2500 mg/kg, iron ≤100 mg/kg, magnesium ≤900 mg/kg and lead ≤5 mg/kg. However, zinc concentration was not reported and thus assumed zero in the analysis.

It has been suggested by Hsiung et al. (1997) that the additional variables should also be included in the identification of the trace metals in living organisms. It can be further stated that if agar is not included then the concentration of zinc in NGM is 0.2 μM. This concentration is less than the 16.15 μM concentration of zinc in the 100 μl LB media. The bacterial strains are concentrating their zinc from the media and the worms obtain their required zinc from bacteria. However, the worms do not take up all of the zinc from bacteria, since they have a lower concentration of zinc than the surrounding bacteria.

The level of Mn, Fe, and Cu were also measured in those samples by ICP-MS. The results of two out of three independent experiments were consistent. Since the results of the 3rd experiment were erroneous due to unexplained reasons as shown in Figure 2-4, when triplicate results were plotted, error bars of 3rd experiment made the results inconclusive. Therefore decision was made not to include those measurements any further, but they are included in the Appendix to show the raw data (Appendix, Figures 2-4).

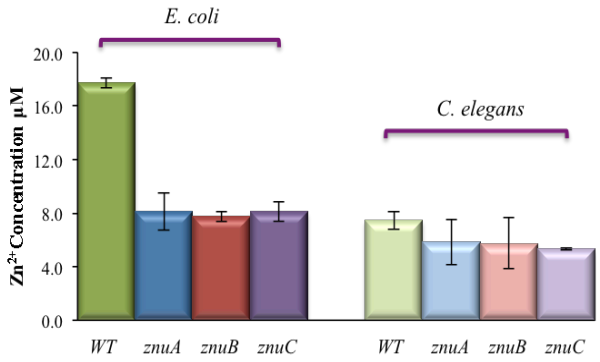

Figure 3.1: Measurement of zinc concentration in 4 strains of bacteria and *C. elegans* fed with the respective bacterial strains. The experiment was carried out in triplicate. WT *E.coli* showed the highest zinc concentration (17.7 µM). Zinc concentration in all the mutant strains was lower (8.1 µM, 7.7 µM, and 8.11 µM respectively, ($p<0.0001$)). Zinc concentration in *C. elegans* fed with the WT bacteria was lower than zinc concentration in WT bacteria 7.5 µM ($p<0.0001$). *C. elegans* fed with mutant showed still lower zinc concentration (5.8 µM, 5.7 µM, and 5.3 µM respectively) than that detected in the respective bacterial strains, although the difference did not reach statistical significance($p>0.05$). Data are presented as means with error bars representing standard deviation (SD).

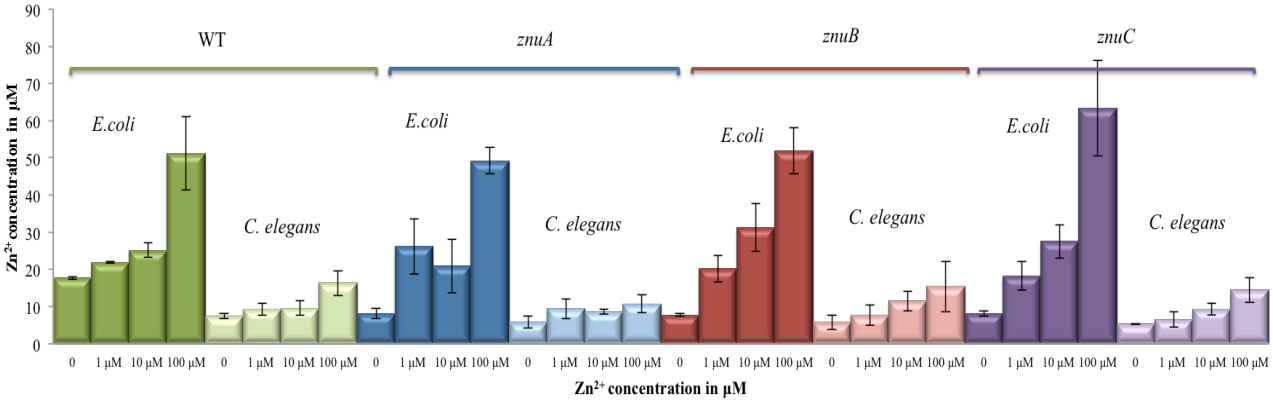

Figure 3.2: Zinc concentration measurements in 4 strains of *E. coli* and *C. elegans* fed with the respective bacterial strains, cultured in NGM media supplemented with indicated concentrations of zinc. The experiments were carried out in triplicates. Increasing zinc in NGM from 0 µM to 100 µM gradually raised intracellular zinc concentration in the wild type strain and the 3 mutant bacteria, and the difference was of statistical significance ($p<0.05$) for all zinc supplementations apart from the concentration range of 1 µM - 10 µM ($p>0.05$). Adding 100 µM external zinc in NGM showed the most pronounced rise in all bacterial strains tested here ($p<0.001$). In *C. elegans* there was a trend towards an increased zinc concentration when worms were fed with bacteria cultured on zinc-supplemented media, whether it was WT strain or the mutant strains, but this increase was not statistically significant for external zinc supplementation in the range of 1-10 µM ($p>0.05$). Only feeding with bacterial strains cultured in NGM with the highest concentration of zinc (100µM) significantly increased zinc concentration in *C. elegans* ($p<0.05$). Data are presented as mean ±standard deviation (SD).

	0 Zn^{2+}		**1 µM Zn^{2+}**		**10 µM Zn^{2+}**		**100 µM Zn^{2+}**	
Bacterial stain	Average	SD	Average	SD	Average	SD	Average	SD
WT	17.7	0.40	22.0	0.25	25.2	1.94	51.2	9.86
znuA	8.1	1.37	26.1	7.37	20.9	7.19	49.2	3.56
znuB	7.73	0.36	20.20	3.57	31.26	6.48	51.96	6.21
znuC	8.10	0.71	18.25	3.82	27.50	4.46	63.44	12.89

Table 3.1 A): Effect of adding different zinc supplements in the NGM on the 4 strains of bacteria.

	0 Zn^{2+}		**1 µM Zn^{2+}**		**10 µM Zn^{2+}**		**100 µM Zn^{2+}**	
Bacterial stain	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
C. elegans on WT	7.47	0.68	9.20	1.61	9.55	1.97	16.26	3.35
C. elegans on *znuA*	5.84	1.69	9.42	2.72	8.61	0.64	10.73	2.38
C. elegans on *znuB*	5.74	1.90	7.66	2.69	11.44	2.58	15.40	6.78
C. elegans on *znuC*	5.33	0.11	6.56	2.07	9.31	1.56	14.46	3.37

Table 3.1 B): Effect of adding different zinc supplements on worms fed with the 4 strains of bacteria.

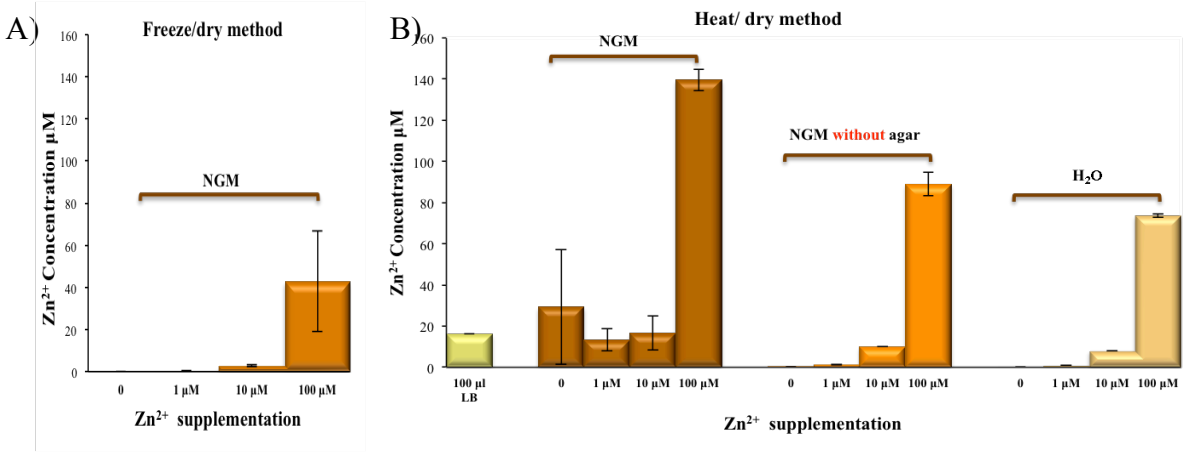

Figure 3.3: Zinc concentration measurement in LB, NGM, NGM without agar, and H_2O using A) freeze dry method, and B) heat dry method. These experiments were done in triplicate. Data are depicted as means ±standard deviation (SD).

	0 Zn^{2+}		1 µM Zn^{2+}		10 µM Zn^{2+}		100 µM Zn^{2+}	
	Average	**SD**	**Average**	**SD**	**Average**	**SD**	**Average**	**SD**
NGM (freeze-dry method)	0.06	0.03	0.57	0.08	2.71	0.55	42.89	23.86
NGM (heat dry-method)	29.26	27.81	13.27	5.35	16.57	8.32	139.64	5.25
NGM without agar	0.16	0.03	1.14	0.03	9.91	0.05	89	5.64
H_2O	0.21	0.01	0.94	0.02	8.07	0.06	73.78	0.91

Table 3.2: Zinc concentration measurements in NGM using two drying methods, NGM without agar, and H_2O, with or without different zinc supplement. NGM with agar in both methods showed very variable and inconsistent data (evidenced by relatively large SD values as compared to averages) as compared to data obtained for H_20 and NGM without agar.

3.2 Lifespan Analysis of *C. elegans* under Different Conditions

3.2.1 Effect of Mutations in Bacterial Zinc Transporter on *C. elegans* Lifespan

Earlier work from our laboratory determined that *znuB* is one of nine genes important for regulation of longevity, as evidenced by the fact that its deletion can increase the lifespan of *C. elegans* (Jia et al*, unpublished data*). These results, combined with the known role of *znuABC* in regulation of zinc transport, led to a hypothesis that low dietary zinc intake in *C. elegans*, achieved by feeding the worms with *E. coli* strains low in zinc, could increase *C. elegans* lifespan. To examine this hypothesis, the effect of genetic mutations in three *E.coli* zinc transporter genes *znuA, znuB*, and *znuC* on the lifespan of *C. elegans,* fed with the respective mutant bacterial strains, was investigated.

Firstly, the lifespan of *C. elegans* fed with the mutant bacteria *znuA* and *znuB*, and worms fed with wild type (WT) bacteria, was determined. Worms fed with *znuB* showed a 12.78% increase in lifespan compared to WT-fed warms, while worms fed with *znuA* did not show any significant difference from worms on WT bacteria (Figure 3.4). These results were similar to results obtained by earlier studies in our lab (Jia et al*, unpublished data*), although a different methodology was employed in this study i.e. worms were left to lay eggs on the WT bacteria and the mutant bacteria were fed from the beginning of life, whilst in previous protocols worms were left to lay eggs on OP50 bacteria, grown until L4 developmental stage, and then transferred to appropriate mutant bacteria. Kanamycin in the media removed any residual OP50 bacteria.

To examine the effect of *znuC* in extending the lifespan of worms, comparison were made among worms fed with *znuB*, *znuC,* and wild type (WT) bacteria. These experiments were performed in triplicate. Worms fed with *znuB* bacteria showed a consistent significant increase in lifespan (10% $p<0.0001$, 8% $p<0.005$, and 13% $p<0.0001$), while worms fed with *znuC* showed some inter-replicate variability in

lifespan increase (8% $p<0.005$, 8% $p<0.0001$ and 2% $p<0.0001$) respectively to the worms fed WT bacteria (Figure 3.5).

3.2.2 Effect of Adding Supplement Zinc on the Lifespan of *C. elegans* Fed with Mutants

To examine the effect of additional external supplement zinc on the lifespan of worms fed with mutant bacteria *znuB* and *znuC*, different concentrations of zinc were introduced in the NGM media (1 μM, 10 μM, and 100 μM). Experiments were performed twice, using the same range of zinc concentrations, and similar results were observed. Feeding worms with *znuB and znuC* bacteria grown in the NGM media supplemented with 1 μM and 10 μM zinc resulted in decreased lifespan compared to worms fed with bacteria from cultures non-supplemented with zinc, and became equal to the lifespan of worms fed with WT bacteria. 100 μM zinc also resulted in decreasing the lifespan of worms fed with the mutant *znuB* bacteria, whereas worms fed with *znuC* showed similar lifespan as worms fed WT bacteria (Figure 3.6 and Appendix: Figure 1).

The results presented in Table 3.3, and Table 1 in Appendix, are not consistent with Figures 3.5, Figure 3.6 A, B, and C. It could be due to the fact that each of these experiments was performed individually at different times. Experiments assessing lifespan were time-demanding, and it was especially difficult to compare different worms fed with different bacteria and different zinc treatments at the same time.

However, these results suggest that adding 1 μM, 10 μM zinc to the NGM media can reverse the effect of the *znuB* and *znuC* in extending worms lifespans. Moreover, adding 100 μM zinc appears to exert a toxic effect on the worms fed with *znuB* bacteria. A particular earlier study (Roh et al., 2012) reported similar toxic effect, using 100- 200 μM zinc supplementation, on the growth rate of *C. elegans.*

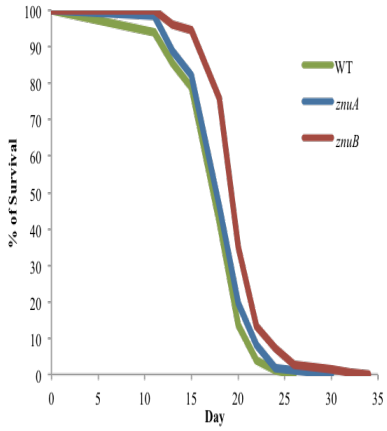

Figure 3.4: Effect of *znuA* and *znuB* mutations in bacterial zinc transporter on *C. elegans* lifespan. Worms fed with *znuB* showed a 12.78% increase in mean lifespan 20.5 days ($p<0.0001$), while worms fed with *znuA* did not show any significant difference from worms on WT bacteria (mean lifespan 18.7 days, and 18.1 days respectively).

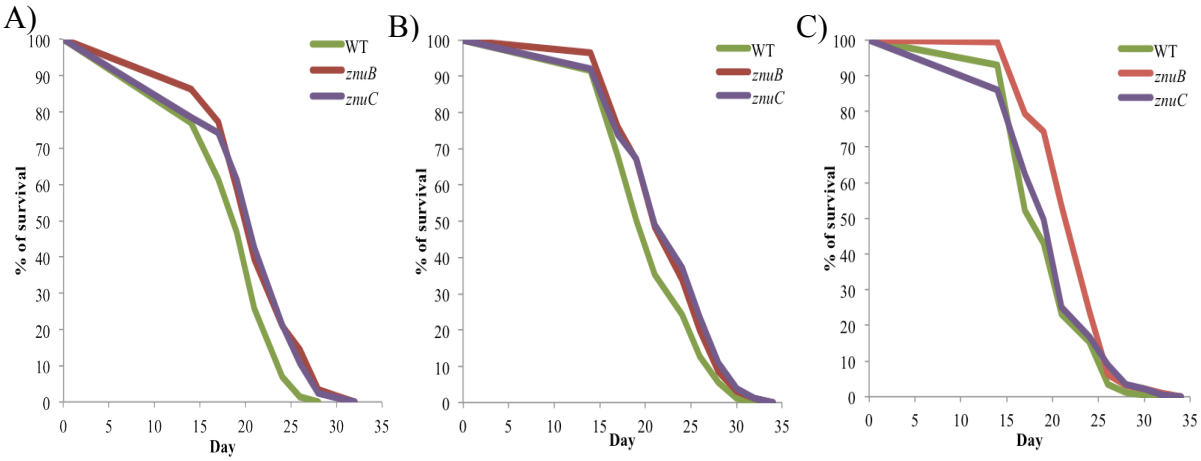

Figure 3.5 (A, B, C): Effect of *znuB* and *znuC* mutations on *C. elegans* lifespan. Triplicate experiments were performed. Worms fed with *znuB* showed a 10% ($p<0.0001$), 8% ($p<0.005$), and 13% % ($p<0.0001$) increase in mean lifespan (21.3, 21.95, and 22.38 days) compared with worms fed with WT bacteria (mean lifespan 19.4, 20.4, and 19.7 days). Worms fed with *znuC* showed 8% ($p<0.005$), 8% ($p<0.0001$), and 2% ($p<0.0001$)

increase in mean lifespan (21.06, 22.08, and 20.21 days) respectively compared with worms fed with WT bacteria (mean lifespan 19.4, 20.4 and 19.7 days).

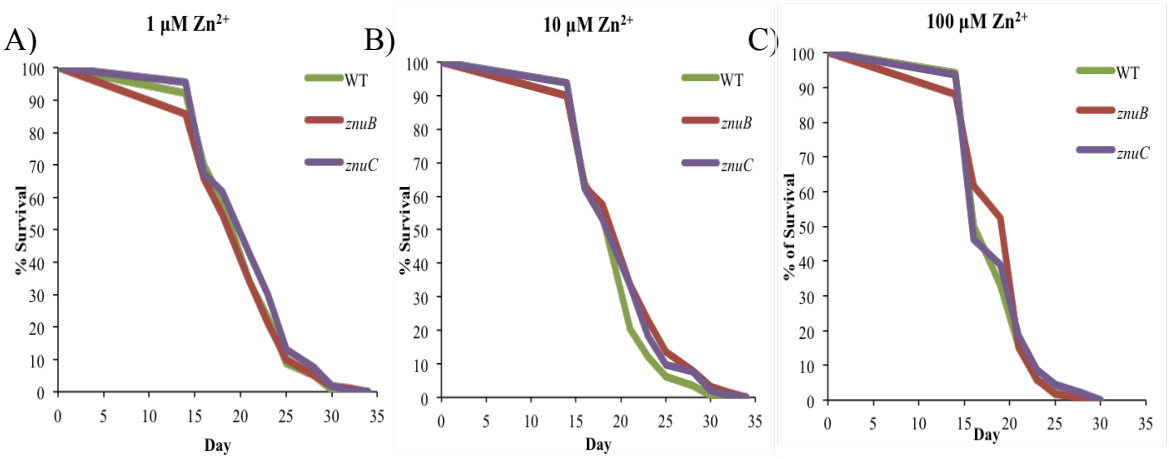

Figure 3.6 (A, B, C): Effect of different zinc concentrations on *C. elegans* lifespan.

Two experiments were carried out at different time, and this figure shows results from one of these replicates. Both of these experiments showed similar results (compare Appendix: Figure 1). The effect of increasing zinc concentration in NGM media on worms fed with *znuB and znuC* bacteria did not result in significant difference in the lifespan of worms fed with WT bacteria ($p>0.1$).

	0 Zn^{2+}	**1 µM Zn^{2+}**	**10 µM Zn^{2+}**	**100 µM Zn^{2+}**
***C. elegans* on WT**	19.4	22.04	21.4	19.1
C. elegans* on *znuB	21.3	22.2	21.7	19.5
C. elegans* on *znuC	21.06	23.0	21.9	19.4

Table 3.3: Summary of the mean of lifespan of *C. elegans* under different zinc concentration in NGM, fed with WT, *znuB*, and *znuC* bacteria. Results in this table show that addition of extra zinc (1 µM -100 µM) had insignificant effect ($p>0.1$).

3.3 Effect of Mutations in the Bacterial Zinc Transporter on *C. elegans* Fecundity

Comparisons were made of the fecundity of wild type (N2) worms fed with the *znuA*, *znuB*, and *znuC* mutant bacteria with the fecundity of worms fed with the WT bacteria at 25°C. Numbers of total progeny produced by worms fed with mutants were much lower compared to the reproduction of worms fed with the WT (33%, 23%, and 70% of the WT values, respectively) (Figure 3.7A). Mutation in the *znuB* had a particularly deleterious effect on worm's fecundity. It was also noticed that feeding C. elegans with *znuB* strain had a strong effect prolonging worm's development into an egg-laying adult as compared to worms fed with other mutant bacteria and wild-type strain (Figure 3.7B).

Apart from comparing the relative number of progeny between *C. elegans* fed with WT and mutant bacterial strains, it is noteworthy to analyse the absolute number of eggs laid by the worms in my experiments. Normally, wild type (N2) *C. elegans* produce around 300 progeny when it is fed with the OP50 bacteria, and used at 20°C. Brood sizes are smaller at 25°C than at 20°C, as shown for example by (Virk et al., 2012), where the mean brood size was around 180 at 25°C. Therefore, worms' fecundity is temperature-sensitive and decreases at high temperatures. In my experiments, I observed an even smaller number of eggs were laid at 25°C (around 77), which may be a result of using a different bacterial strain, namely Keio collection K12 derived strain.

Adding 10 μM zinc to the NGM media restored the number of progeny produced by worms fed with the *znuA* and *znuC* mutants. It had little effect, however, on worms fed with the WT bacteria (Figure 3.7A). There was also a trend towards restoration of progeny production in worms fed with znuB grown in media

supplemented with 10 μM zinc, but this effect was not as pronounced as in worms fed with the other two mutant strains. Zinc supplementation did not rescue the time delay in fecundity for worms fed with *znuB* (Figure 3.7C). These results support the importance of zinc in animal fertility (Clapper et al., 1985). Liu et al. (2013) showed that in *C. elegans* zinc is sufficient to trigger sperm activation *in vitro*, and that extracellular zinc induces the intracellular redistribution of labile zinc.

To test whether another gene was mutated in the bacterial strains used in the study, an experiment was performed to compare the fecundity of worms fed with *znuB*, complemented *znuB* bacteria (rescued with plasmid encoding for wild-type *znuB*) and compared it to worms fed with WT bacteria. This experiment was performed to determine the possibility of mutation of any other gene in the *znuB* bacteria which could have caused variations in the transmembrane permeability of the bacteria affecting the transport of the supplemented zinc resulting in decreased zinc uptake and subsequently lower level of fecundity of worms. If there was an improvement in the fecundity of worms fed with WT bacteria or complemented *znuB* bacteria then it would suggest that decreased fecundity of worms is attributed to the mutation in *znuB* gene and not related with any other gene.

Carbenicillin was included in the NGM media to maintain the complementing plasmid and a control plasmid in the control strain. The total progeny of worms fed with *znuB* were much lower (33%) as compared to worms fed with WT strain, but the time delay in the egg production in these worms was not seen in this experiment. Worms fed with complemented *znuB* produced more progeny, around 75% of the values obtained for *C. elegans* fed with WT bacteria (Figure 3.8)

These data confirmed that the observed loss of fecundity in *C. elegans* fed with *znuB* mutant strain was indeed caused by mutation in *znuB*, rather than other genes, and

in terms of pathogenesis this effect was caused by abnormal transmembrane zinc transport in the mutant bacteria leading to decreased zinc uptake and concentration. Therefore, the worms fed with *znuB* bacteria produced fewer progeny as compared to the worms fed with WT bacteria, because the WT bacteria had the phenotypic *znuB* gene which ensured zinc uptake from the media and supported the fecundity of worms which were fed upon it. A significant increase in the progeny of worms fed with the genetically complemented *znuB* bacteria suggests that *znuB* is important for zinc uptake from the surroundings by the bacteria, and presents the evidence that dietary zinc concentration affects worm's fecundity but is not responsible to cause significant effect on the duration of worm's reproduction.

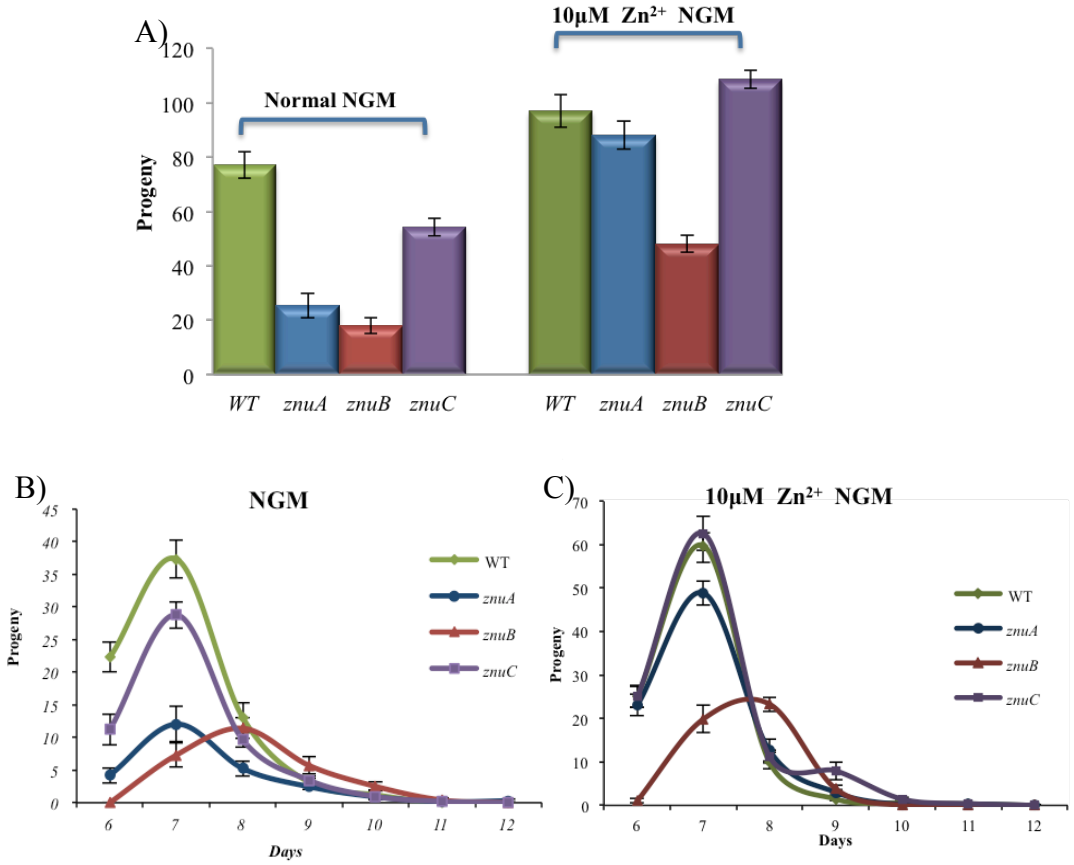

Figure 3.7: Effect of mutations of the bacterial zinc transporter on *C. elegans* fecundity. (Experiment was done twice, 10 worms per each bacterial strain)**.**

A) The fecundity of worms fed with mutants was lower compared to worms fed with WT (n= 25, 18 and 48 *versus* WT n=77 respectively; ($p<0.0001$)). Adding 10µM zinc to the NGM plates increased the fecundity in worms fed with *znuA* and *znuC* bacterial strains (n= 88 and n=108, respectively) compared to worms fed with WT (n=97). 10 µM zinc increased fecundity in worms fed *znuB* (n=48) but not to the same extent as the increase observed worms fed with the other mutants and WT bacteria ($p<0.0001$). Error bars are ±standard deviation (SD).

B & C) Worm egg laying per day in NGM plates with and without zinc supplementation. **B)** Worms fed with *znuB* showed a time delay in starting egg production compared to worms fed with other mutants and WT bacteria. **C)** Adding 10 µM zinc increased egg laying in worms fed with *znuB*, but did not affect the time delay. Error bars are ±SD.

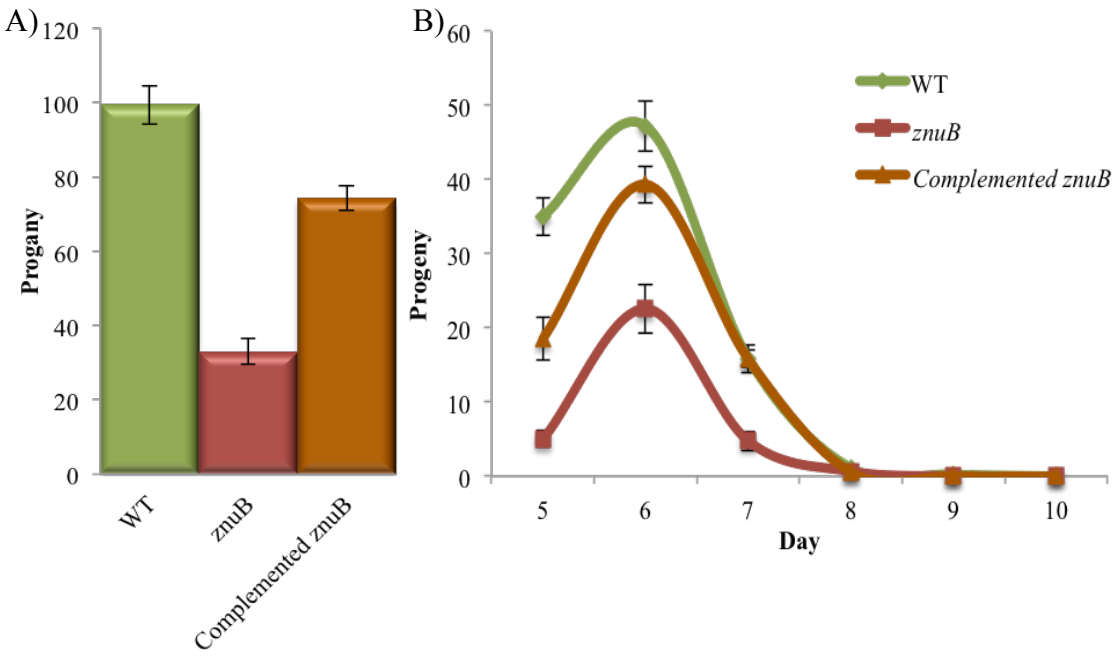

Figure 3.8: Effect of *znuB* mutation and its rescue on *C. elegans* fecundity. (Experiment was done twice, 10 worms per bacterial condition). A) Fecundity of worms fed with *znuB* was lower than worms fed with WT (n=33 and n=99, respectively, ($p<0.0001$)). The number of progeny from worms fed with bacteria complemented for *znuB* with a rescue plasmid was close to worms fed with WT bacteria (n=75 ($p<0.0002$)). B) Worms fed with znuB did not show any time delay in production comparing to worms fed with WT and complementing *znuB*.

3.4 Imaging Developmental Rate

We performed imaging of SS104 *C. elegans*, grown at 15°C and fed with the 3 mutant bacterial strains *znuA, znuB*, and *znuC*, and compared it with worms grown on WT bacteria ±supplemented zinc. We started taking images from day 5 of life cycle (L3/L4) until worms reached day 13 of their life cycle (deemed as adulthood). Despite lack of quantification, the images show clearly that the worms grown on *znuA* and *znuC* mutant strains were developing in a normal manner, comparable to worms grown on WT bacteria ± zinc supplementation, between day 5 and 13. Worms fed with *znuB* mutant bacteria showed, however, an obvious developmental delay within the same time frame (Figures 3.9-3.13).

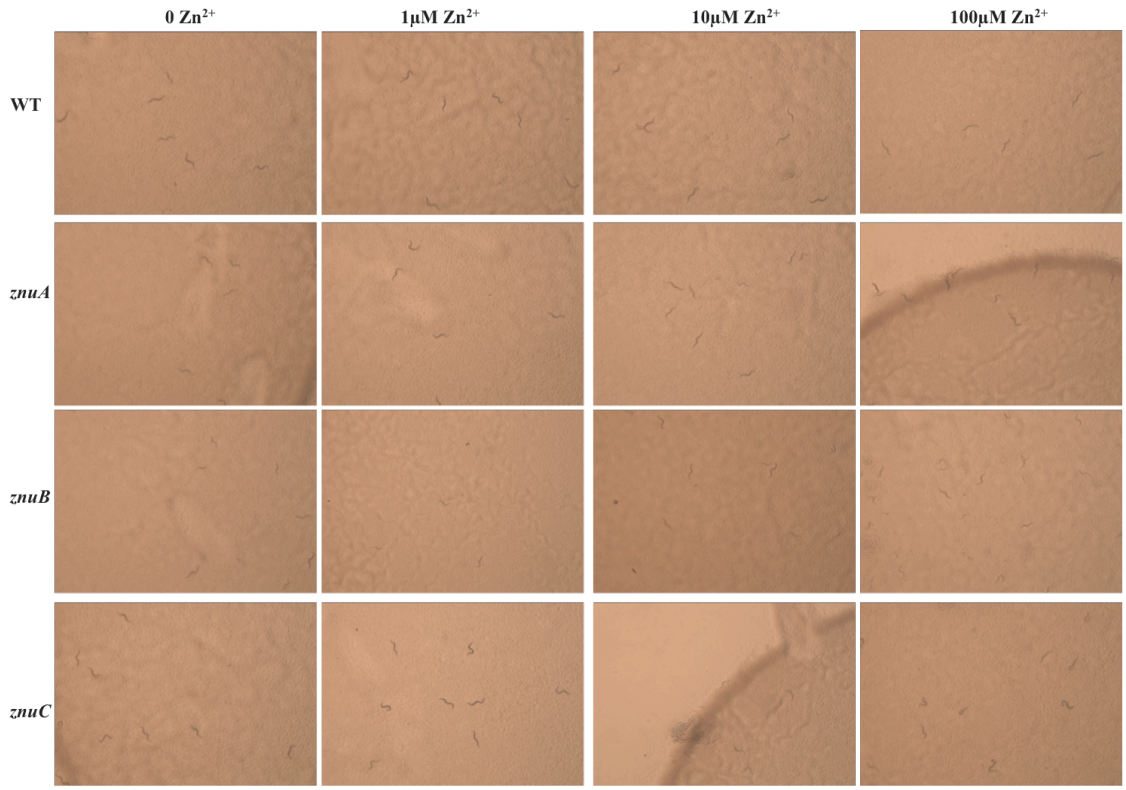

Figure 3.9: Day 5 of lifespan.

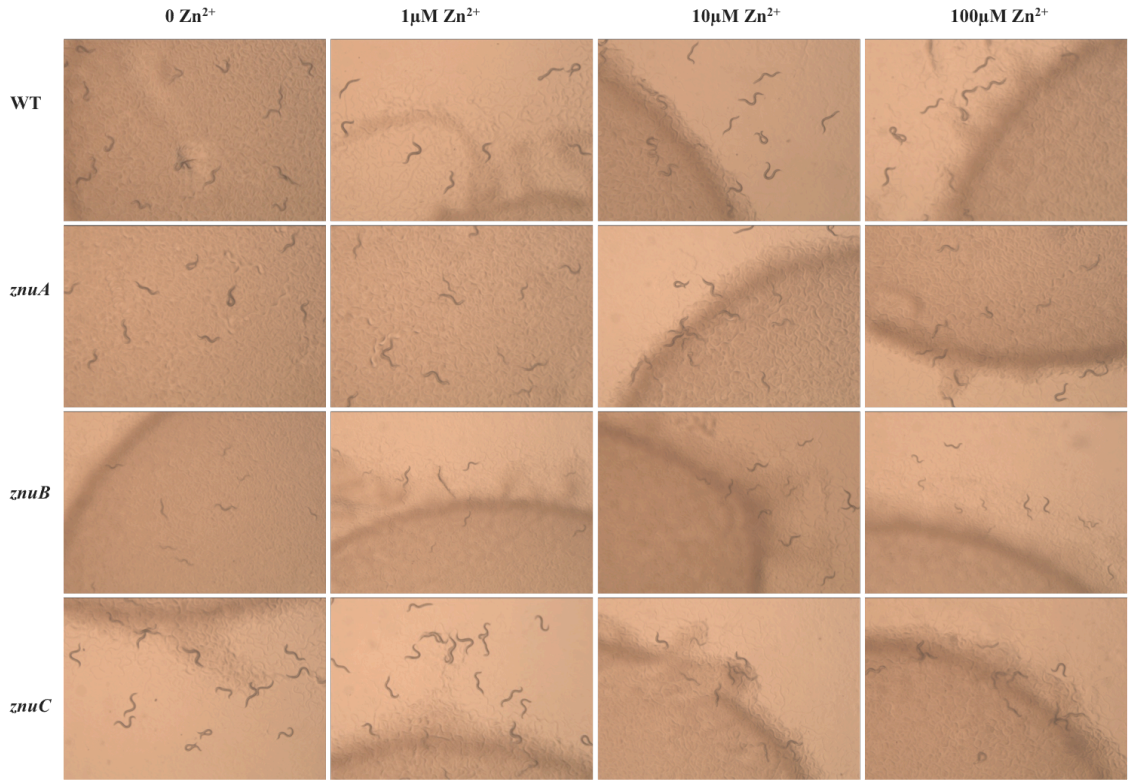

Figure 3.10: Day 7 of lifespan.

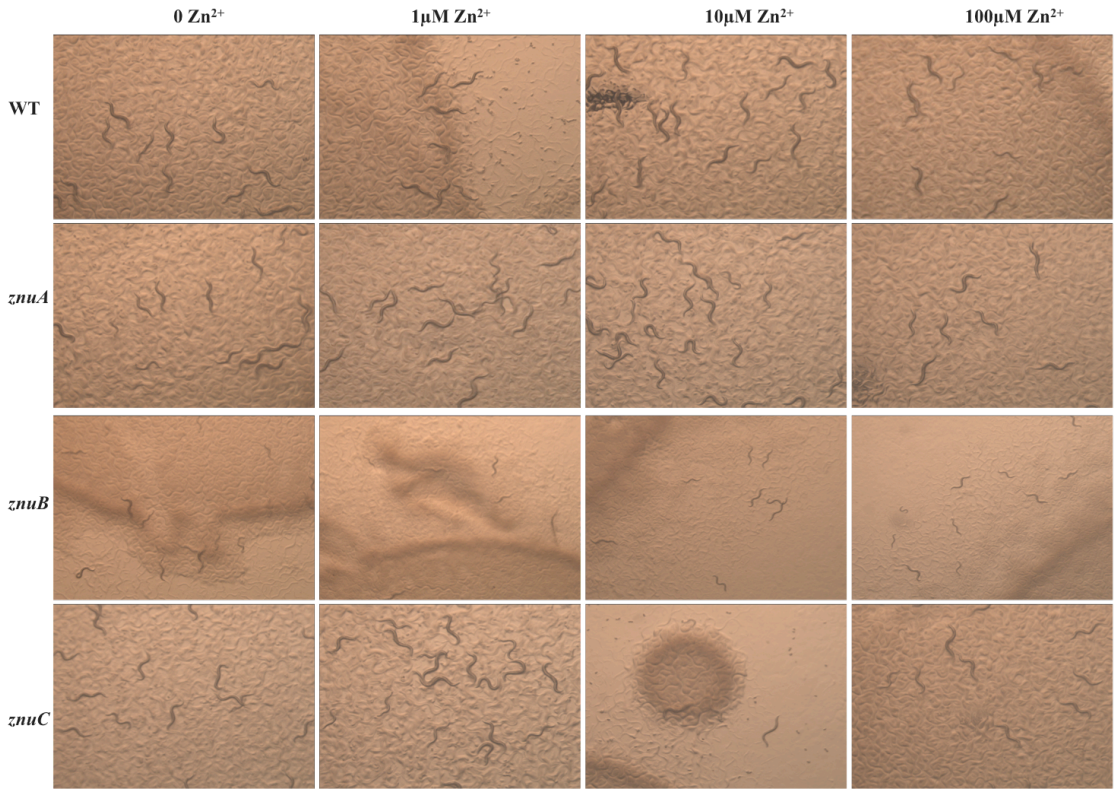

Figure 3.11: Day 9 of lifespan.

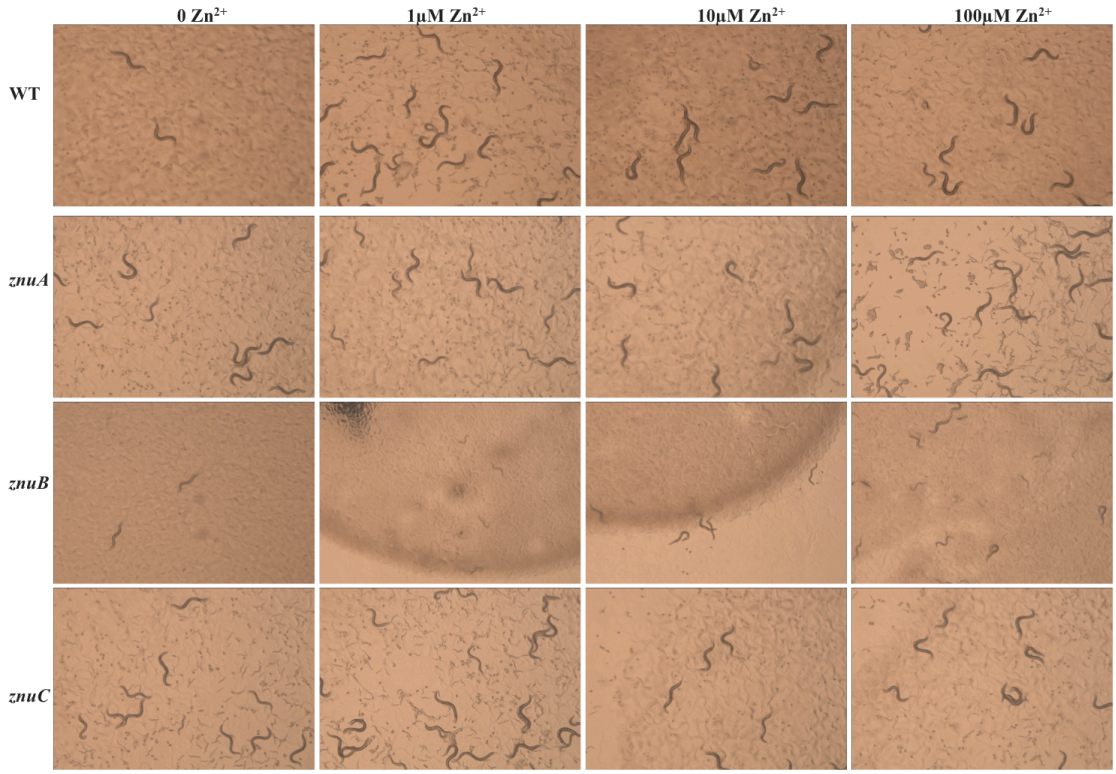

Figure 3.12: Day 11 of lifespan.

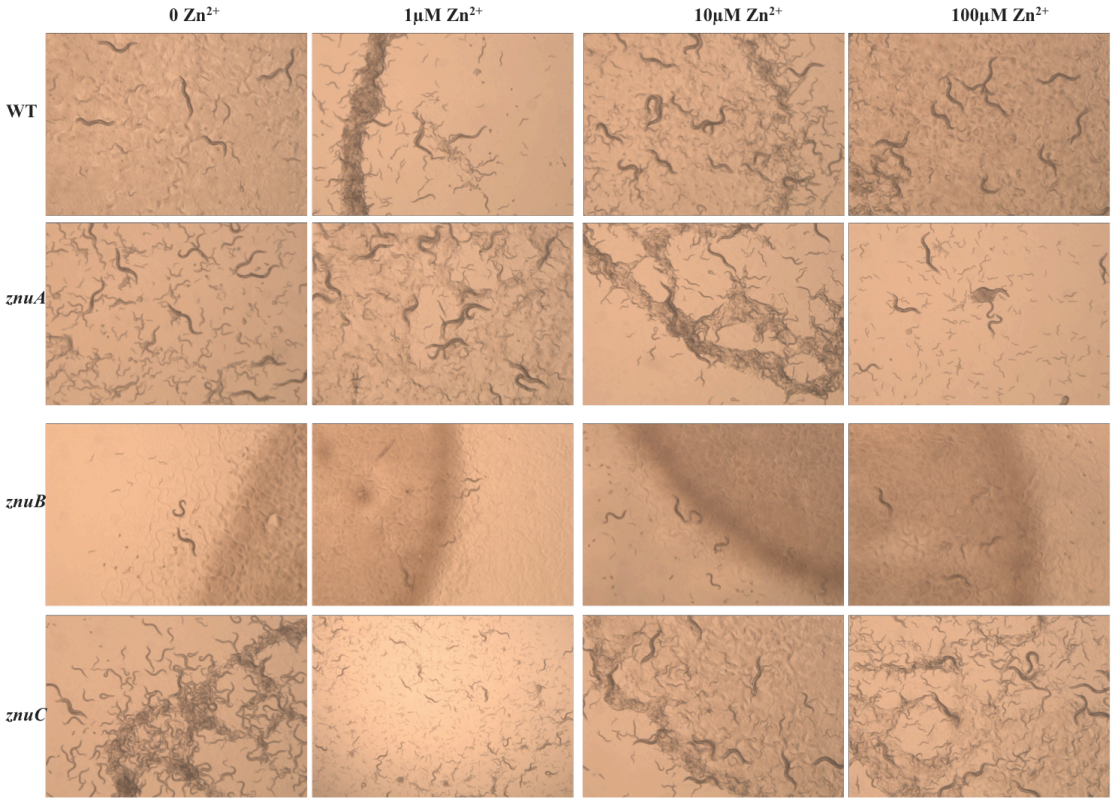

Figure 3.13: Day 13 of lifespan.

CHAPTER 4: DISCUSSION

A wealth of information obtained through concerted research efforts and spanning many years of work, is available to show the importance of iron in host-microbe interaction. The pivotal role of another microelement, zinc, in such interaction has only recently gained the attention of research community. Recently, it has been reported that deletion of the *znuABC* genes not only decreases the ability of bacteria to grow in zinc deficient environment *in vitro*, but also severely compromises their pathogenicity (Cerasi et al., 2013). Building upon these observations, we hypothesised the microbial zinc concentration plays an important role in the host-microbiota physiology. We used *C. elegans* as a eukaryotic model system to study the host-microbe interaction in the presence and/or absence of zinc. Bacterial strains from the Keio collection were used, with a set of single-gene deletion mutants of *E. coli* K-12 established, as described previously (Baba et al., 2006). Since deletion of *znuB* gene in *E. coli* was identified to be associated with an increase in *C elegans* lifespan (Jia et al, unpublished data), in this study we aimed to determine the impact of feeding worms with bacteria harbouring single mutations in *znuABC* genes on worm zinc concentration and their ageing process (using lifespan and fecundity as surrogate measures of ageing).

As described earlier (see section 1.4.3), *znuABC* is the high-affinity transporter system, consisting of 3 transporting proteins: *ZnuA,* which is the periplasmic component*, ZnuB*, which acts as a transporter through the plasma membrane, and *ZnuC,* which facilitates ATP hydrolysis that provides energy required for zinc transport (Cerasi et al., 2013).

In the present study, we analysed the effects of feeding *C. elegans* with three

specific mutant bacterial strains *(znuA, znuB,* and *znuC)*, as compared to the wild-type strain, on the zinc-associated changes in worm's ageing, measured with such surrogate markers as intracellular zinc concentration (in the bacteria and in the worms themselves), worm development, ability to lay eggs, and lifespan.

4.1 Effect of the Zinc Concentration in the *E. coli* Mutants on *C. elegans* Zinc Concentration

Zinc concentration in the worms and mutant bacteria they were fed with (*znuA, znuB*, and *znuC* mutants) were measured, and compared to the WT bacteria fed worms and WT bacteria, by using ICP-MS. The calculations were based on weight of the bacterial and adult worm pellets. Results showed that the WT bacteria had a higher intracellular concentration of zinc compared to all 3 mutant strains, which is in line with the previously reported cellular function of *znuABC* transporter system. With regards to zinc concentration in *C. elegans*, it was similar irrespectively of which bacterial strain was used as a food source.

Zinc concentration in LB and NGM media was also measured using the same approach (ICP-MS), in order to establish the baseline level of zinc. Measurements of zinc concentration in the NGM media were not consistent, however, and by repeating the measurements with varying solutions we established this lack of reproducibility was due to the matrix effect, introduced most likely by the presence of agar in the NGM media. Based on the element analysis, carried out in agar using ICP-OES, the manufacturer reported absence of zinc in the NGM media. Noteworthy, Hsiung et al. (1997) has emphasised the importance of non-spectral and spectral interferences during analysis of elements in biological matrices in his study.

It can be further stated that if the matrix effect of agar is excluded, and it is

assumed that there is no zinc in the agar; zinc concentration in NGM is ~0.2 μM. This zinc concentration is less than that in LB media (16 μM) on which WT bacteria are cultured. Deletion of *znuA, znuB*, and *znuC* genes in mutant bacteria resulted in 50% decrease in zinc concentration compared to zinc concentration in WT bacteria. Nevertheless, the worms fed with either WT or mutant bacteria showed statistically insignificant difference in zinc concentration.

Yang et al. (2006) showed that the extracellular zinc concentration plays a very important role in the growth of the *znuA* mutant. In addition, Ammendola et al. (2007) reported, using a pathogenic bacteria *Salmonella enterica*, that ZnuA plays a role in maintaining the ability of the bacteria to multiply in the phagocytes, and accordingly that loss of ZnuA function correlates with lower pathogenicity of the mutant strains. Results from the same study further showed that the prokaryotic *znuABC* transport system plays a role in zinc uptake when bacteria are challenged with reduced zinc concentrations in their environment (Ammendola et al., 2007). Moreover, there are certain endogenous zinc pools which are sensitive to the depletion of zinc. The *znuABC* mutants can still survive, providing that high external concentration of zinc is available. Further studies (Lewis et al., 1999) have suggested that the infected animals have physiologically reduced amount of the metals, which are necessary for the bacterial growth. In addition, it has been suggested that the increased concentration of zinc in the tissues is maintained by the *znuABC* transporter system, although this remains to be proven.

Zur (zinc uptake regulator) regulates the *znuABC* genes in *E. coli.* When extracellular zinc is present in sufficient amount, Zur blocks the expression of the *znuABC* genes (Davis et al., 2009b). Therefore, levels of *ZnuABC* are regulated via a negative-feedback loop involving sensing the availability of zinc in the extracellular environment.

The results from above mentioned studies provide a conceptual framework to explain my results which show that increasing extracellular zinc concentration in NGM media leads to a corresponding significant increase in the intracellular zinc concentration in the 3 bacterial mutants (Table 3.1 and 3.2). Despite this, worms fed with the 3 mutants bacterial strains, cultured on media supplemented with 1 μM and 10 μM external zinc, showed an insignificant increase in zinc concentration as compared to worms fed with bacteria cultured on normal (non-supplemented) NGM media. This changed with higher external zinc supplementation (100 μM) in NGM media, with zinc concentration in worms fed with the 4 bacterial strains rising two fold as compared to the worms fed with 4 bacterial strains cultured in normal NGM media. Nevertheless, zinc concentration in these worms remained lower than zinc concentration in the respective bacterial strains they were fed with, even in the presence of 100 μM external zinc in NGM media. These results indicate that worms obtain zinc required for their physiological process from the consumed bacteria, and suggest therefore that such experiments can increase our understanding of how bacterial zinc levels influence the zinc level in *C. elegans.* Obtaining a better picture on zinc level regulation by microbiota will also shed light on the mammalian host- microbiota interaction. To the best of my knowledge this study is the first to examine how the level of zinc in bacteria, regulated by introducing genetic mutations that affect the functioning of the *znuABC* transporter system, and used as a food source to *C. elegans* as a model system of animal/human physiology, affects worms in terms of their development and aging.

Davis et al. (2009a) were the first group to report zinc content in *C. elegans*, showing that total animal zinc content is related to the concentration of dietary zinc. In that particular study the authors have shown an interesting relationship between dietary zinc and zinc content, whereby sensitivity of the responsiveness to changes in dietary zinc diminished with increasing dietary zinc content (i.e. showed an inverse correlation

with dietary zinc level). The study suggests that low dietary zinc concentration either stimulates zinc uptake or minimises zinc excretion (with net result of counterbalancing the environmental shortage), whereas higher dietary zinc concentration leads to maximum zinc content, so that animals respond to any further increase in dietary zinc with a minimal increase in zinc content (i.e. the system reaches saturation level).

Davis et al. (2009a) reported in their study that the optimum concentration of zinc (0.25 μM) in *C. elegans* Maintenance Medium (CeMM), rather than the NGM media, is desirable for the normal worm growth. In my current study, I achieved the similar zinc content with metal analysis in NGM media, with the matrix effect exclusion (~0.2 μM). The reason Davis et al. (2009a) chose CeMM media was the difficulty in zinc solubility in the NGM media when using $ZnCl_2$ and $ZnSO_4$. I did not encounter that problem using $ZnSO_4.7H_2O$ in my experiments.

Several authors, including Davis et al. (2009a), Murphy et al. (2011), Roh et al. (2012), and Roh et al. (2013), also measured zinc concentration in WT worms fed with OP50 bacteria and compared them with mutant worms to understand basics of zinc metabolism in *C. elegans*.

Table 4.1 summarises the methodology and results from other studies, providing a comparison to my current study. These studies differ from mine with respect to the type of media, worm and bacterial strain used, and the fact that worms collected for zinc measurements were 3 weeks old cultured worms (i.e. from all the worms life stages, including eggs, L1, 2, 3, 4 young adults, adults and old worms, even died worms). In my study, I used SS104 *glp-4* (*bn2*) *C. elegans* strain for zinc measurement and lifespan experiments, since this strain has a temperature sensitive fertility phenotype. It is fertile at 15°C and it is infertile at 25°C, and thus performing experiments at 25°C (infertile worms) are easier and more precise with regards to measuring zinc concentration in adult (day 9 of lifecycle) worms.

Results obtained by the previous studies were different from my results, because the way calculations were carried out to determine the concentration of zinc i.e. dividing ICP-MS results by the *C. elegans* dry weight. According to (Teramoto et al., 2010) *C. elegans* dry weight is estimated to be 20% of the wet weight. To compare my results to previous studies I recalculated my data to normalise by dry weight by dividing the results by 0.2 since dry weight of *C. elegans* is 20% of the wet weight.

Table 4.2 summarises zinc concentration obtained in previous studies for different strains of worms grown on OP50 bacteria, and zinc concentration of current study obtained in SS104 strain worms fed with the 3 mutants bacterial strains and WT bacteria, expressing results in ppm (parts per million). Previous studies indicated that zinc concentration in worms increases with increasing the environmental zinc concentration. The discrepancy between these results and the data obtained in my study could be related to the differences in methodology (including bacterial strain, type of media used). Secondly, another factor affecting the result obtained in this and previous studies are the biological differences between the C. elegans strains, including their ageing, and quantity and the concentration of zinc in the worms used in my study.

Method obtained	Zinc	Media	Reason	Supplementation of zinc used	*C. elegans* strains	Bacterial strain
Davis et al. (2009a)	$ZnCl_2$	CeMM	The CeMM media was used due to zinc solubility problem	Ranged 6 µM- 2 mM	WT (N2), *cdf-2*	OP50
Murphy et al. (2011)	$ZnCl_2$	CeMM		0.075 mM, 1 mM	WT (N2), *am130*	OP50
Roh et al. (2012)	$ZnSO_4$	NAMM	The NAMM media was used to prevent bacterial growth	0, 200 µM	WT (N2), *pgp-2, glo-1 & cdf-2*	OP50
Roh et al. (2013)	$ZnSO_4$	NAMM		0, 200 µM	WT (N2), *ttm-1 & cdf-2*	OP50
Current study	$ZnSO_4.7H_2O$	NGM	I did not face any zinc solubility problem	0, 1 µM, 10 µM, & 100 µM	SS104 (*glp-4*(*bn-2*))	Keio Collection: WT, *znuA, znuB & znuC*

Table 4.1: Comparison between methods performed previously and current study to measure zinc concentration in *C. elegans.*

Results Obtained by	*C. elegans/ E. coli*	Zn^{2+} concentration in ppm	Zn^{2+} supplementation in media							
			0 µM	1 µM	6 µM	10 µM	75 µM	100 µM	200 µM	1 mM
Davis et al. (2009a)	WT/ OP50		N.G	N.G	40	N.G	N.G	100	N.G	550
	cdf-2/ OP50		N.G	N.G	30	N.G	N.G	80	N.G	200
Murphy et al. (2011)	WT/ OP50		N.G	N.G	N.G	N.G	N.G	250	N.G	1500
	am130/ OP50		N.G	N.G	N.G	N.G	N.G	250	N.G	1500
Roh et al. (2012)	WT/ OP50		125	N.G	N.G	N.G	N.G	N.G	250	N.G
	pgp-2/ OP50		100	N.G	N.G	N.G	N.G	N.G	200	N.G
	glo-1/ OP50		90	N.G	N.G	N.G	N.G	N.G	150	N.G
	cdf-2/ OP50		90	N.G	N.G	N.G	N.G	N.G	100	N.G
Roh et al. (2013)	WT/ OP50		150	N.G	N.G	N.G	N.G	N.G	500	N.G
	ttm-1/ OP50		170	N.G	N.G	N.G	N.G	N.G	650	N.G
	cdf-2/ OP50		100	N.G	N.G	N.G	N.G	N.G	180	N.G
Current Study	SS104/ WT		2.44	3.01	N.G	3.12	N.G	5.3	N.G	N.G
	SS104/ *znuA*		1.91	3.08	N.G	2.81	N.G	3.51	N.G	N.G
	SS104/ *znuB*		1.88	2.5	N.G	3.74	N.G	5.03	N.G	N.G
	SS104/ *znuC*		1.74	2.14	N.G	3.05	N.G	4.73	N.G	N.G

Table 4.2: Results from current study in comparison to results obtained previously. (N.G: not given).

4.2 Effect of *E. coli* Mutants on *C. elegans* Lifespan

It is evident that every organism experiences loss or reduced biological functions over time, a phenomenon referred to as progressive ageing (Muschiol et al., 2009). The total rate of ageing can be easily identified by the lifespan of the organism. When considering the nematodes, lifespan is explained as the number of days in which the worms are able to respond to the stimuli (Sutphin and Kaeberlein, 2009).

I studied the effect of zinc on *C. elegans* lifespan by feeding the worms with bacteria harbouring zinc transporter mutations (i.e. *znuA, znuB*, and *znuC* mutant bacteria). The lifespan of worms fed with mutant bacteria was compared with that of the worms fed with the WT bacteria. The experiments were performed in triplicate, and the results showed an average of 12% and 6% increase in the lifespan of *C. elegans* fed with *znuB and znuC* strains, respectively, as compared to the *C. elegans* fed with WT bacteria. The worms that were fed with *znuA* did not show, however, any major differences in lifespan as compared to the worms fed with WT bacteria.

It was suggested in an earlier study (Berducci et al., 2004) that in the periplasm, ZnuA has a noticeable effect on maintaining stable zinc concentration by pulling zinc from other zinc metalloproteins, such as Cu,ZnSOD (superoxide dismutase). The study also suggested that changing the periplasmic zinc balance would not affect the amount of free zinc able to enter the cytoplasm by other zinc and nonspecific divalent metal importers. This could explain why there was no effect of mutation in *znuA* on the lifespan of *C. elegans*, as it suggests that *znuA* might not have a significant effect on cytoplasmic zinc, as in the case of *znuB* and *znuC*. It only temporarily holds zinc that is meant to be delivered to *znuB* in the plasma membrane.

In the second part of this study I also investigated the effect of supplemented zinc on the lifespan of *C. elegans*. Different concentrations of zinc were added to the NGM media, and again the bacteria cultured on such supplemented media was used as dietary source of zinc for *C. elegans*. The results, obtained from two independent experiments, suggested that increasing the exogenous concentration of zinc (from 1 μM to 10 μM) in NGM media could reverse the effect of the *znuB* and *znuC*, responsible for enhancing the lifespan of worms.

These results are supported by the fact that when external zinc concentration is high, the *Zur* system acts to down-regulate *znuABC* transporter (Davis et al., 2009b). Moreover, addition of 100 μM of zinc to the media had a toxic effects on the worms fed with *znuB* mutant. Their lifespan was shorter than worms supplemented with 1 μM and 10 μM zinc. A study by (Davis et al., 2009a) also showed that worm maturation was delayed with increasing zinc supplementation (in the concentration range from 2 μM to 500 μM), and high zinc concentrations resulted in significant growth impairment of the worms. In the present work, adding zinc in the range of concentrations between 1 μM and 100 μM to worms fed with *znuB* bacteria showed obvious worm developmental delay between day 5 and day 13. These data were obtained by visual inspection of un-quantified images (Figure 3.9-3.13).

Another study also showed that 100- 200 μM of zinc supplementation in the media results in toxic effects on the growth rate of *C. elegans* (Roh et al., 2013). The results of these studies are consistently suggesting the importance of *znuB* and the critical role it plays in transporting zinc through plasma membrane.

4.3 Fecundity of *C. elegans* Influence by the Bacterial Mutation

The fecundity of the wild type (N2) worms feeding on the *znuA*, *znuB*, and *znuC* mutant bacteria was analysed, and compared with the worms which were fed with the WT bacteria, in the NGM media along with kanamycin at a temperature of 25°C. The total progeny number produced by the worms that were fed with the mutant bacterial strains, particularly *znuB* mutant strain, was low when compared to worms fed with the WT bacteria. These results support the role of *znuABC* transporter, and in particular *znuB* component, in regulating fecundity of *C. elegans*. Earlier studies addressing the topic of reproduction in mammals also showed compromised fecundity in relation to zinc deficiency, and the pathogenesis of such changes can be explained by the fact that a lot of zinc proteases are involved in the process of fertility (Carpentier et al., 2004; Hadwan et al., 2012).

I next investigated the effects of zinc supplementation in worm's fecundity. When 10 μM of zinc was added to the NGM media, it restored the number of progeny produced by the worms fed with *znuA* and *znuC* mutant bacterial strains. The worms were fed with the *znuB* strain showed the potential restoration of zinc. The supplementation of zinc did not rescue the delay in time to reach an egg-laying developmental stage of the worms that were fed with the *znuB* mutant strain.

In the recent years studies have showed the importance of oral zinc supplementation in restoring zinc in seminal plasma of unfertilised patients (Hadwan et al., 2012). In addition, Liu et al. (2013) indicated that zinc is important for the activation of sperm *in vitro* and that zinc can cause the redistribution of the labile zinc. Similarly, a study by Abed (2013) has shown that zinc is necessary for the process of spermatogenesis.

Another experiment performed in this study was to determine the fecundity of worms that are provided with the bacteria carrying mutation of *znuB and* bacteria carrying a complementing *znuB* plasmid (rescue plasmid) as compared to the bacteria that are fed with the WT bacteria in the NGM media, which also had carbenicillin. The worms fed with the *znuB* mutant strain had a reduced total progeny as compared to the worms that were provided with the WT bacteria. Interestingly, there was no delay in the time to reach an egg-laying developmental stage. The worms that were provided with the complemented *znuB* bacterial strain displayed an increased number of progeny, reaching approximately 75% of that observed in worms fed with the WT bacteria. Based on these results we can infer that the mutation in the bacterial *znuB* gene exerts profound effects on warm fecundity, and upon genetic restoration of functional *znuB* with a rescue plasmid the worms are able to largely restore their fecundity, reaching levels similar to the worms fed with the WT bacteria.

CHAPTER 5: CONCLUSION AND FURTHER WORK

The main aim of this study was to identify the effect of *znuABC* zinc transporters in *Escherichia coli* on ageing in the nematode *Caenorhabditis elegans*. The results of 3 independent experiments showed that the *znuB* mutant bacterium has a significant effect on decreasing zinc concentration not only in *E. coli* but also in *C. elegans* fed with the mutant strain. It is worth noting that my results are similar to another study from our lab, investigating the role of folates on worm lifespan, which identified that the warm lifespan is determined not by the worm folate concentration *per se* but rather by the bacterial folate concentration (Virk et al., 2012). Mutation of *znuB* in bacteria showed a significant effect on increasing worm lifespan, and decreasing their fecundity and production starting point.

The results further showed that worms grown on *znuB* mutants displayed a delay in developmental and reproductive functions, between day 5 and 13, as compared to worms fed with the other mutants and WT bacteria, lending further support to the results obtained with the analysis of lifespan and fecundity.

The *znuB* gene plays a very important role in the zinc transporting system *znuABC*, and more specifically in transporting zinc through plasma membrane in zinc-deficient environments. Deletion of the *znuB* gene resulted in delaying worm maturation and decreasing their fecundity, but at the same time increased the worm lifespan.

The current results have raised questions about the role of *znuB* in transporting the hydrophilic zinc ion through plasma membrane, and its importance for interaction between the host and pathogen which needed to be further investigated. Much of the earlier research has focused on *znuA* and its involvement in the host/ pathogen

interaction. These studies have highlighted the importance of the *znuA* gene as a factor for survival and immunogenicity of *E. coli*, *Brucella abortus*, *Haemophilus spp*., *Neisseria gonorrhoeae*, and *Pasteure llamultocida*, and a factor responsible for insufficient zinc level within mammalian hosts (Lu et al., 1997; Patzer and Hantke, 2000; Canny and McCormick, 2008; Davis et al., 2009b). The results of my study anticipate that more research needs to be carried out to elucidate the functions of *znuB* in the host-microbiota interaction.

Considering the previously reported observations, we envisage that further exploration of the mechanisms involved in the regulation of host ageing by bacterial zinc is required before we can develop a thorough understanding of this physiological process. The effect of mutation in the bacterial *znuB* gene on increase in the lifespan of the worms was due to lack of availability of zinc caused by to mutation, which consequently lead to decreased concentration of zinc in the worms feeding on the mutant bacteria. Therefore, we can infer that zinc transporters of *E. coli* can influence the process of ageing in *C. elegans* because these transporters are essential for the uptake and transport of zinc in the bacteria, and in turn the dynamics of microbe-host interaction is affected by the decreased availability of zinc experienced by the bacteria. Reduction of intracellular zinc concentration in mutant bacteria influences the reproduction capability of the worms fed with these bacteria, which ultimately results in storage of the available zinc by the worms and slowing of their development. Consequently, the age of the worms increases. This study has formed the basis on which further exploration of the cellular, biochemical and molecular mechanisms linking the function of zinc transporters of *E. coli* with the ageing of *C. elegans* can be built upon by other researchers. Therefore, further research is needed to validate the results of this study, particularly considering that this is the first study to ever investigate the role of zinc transporters of the *E. coli* on the ageing of *C. elegans*.

This study has increased our understanding of the host-microbe interactions and in particular understanding of the effects that variations in the microbial zinc concentration exert on the ageing process of the host.

APPENDIX

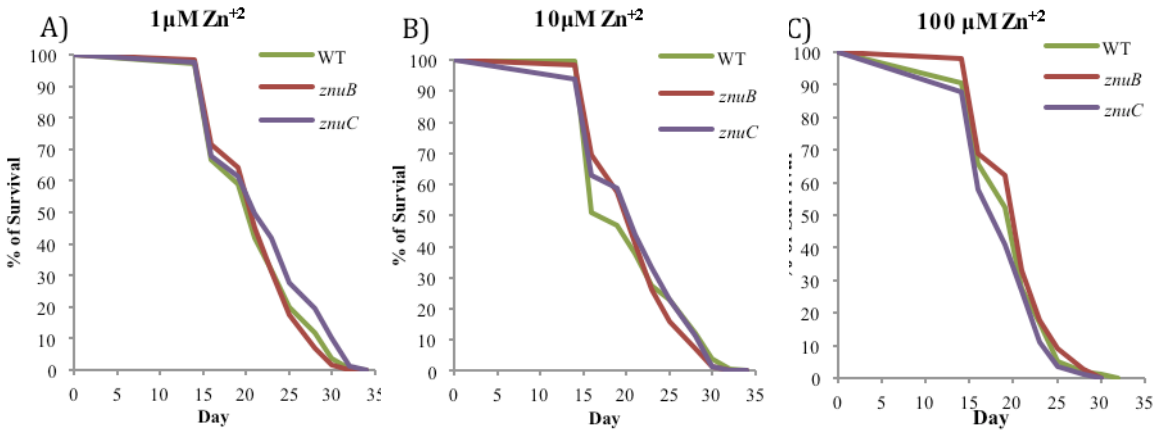

Figure 1 (A, B, C): Repeating the experiment of the effect different zinc concentration on *C. elegans* lifespan fed with mutants and WT bacteria.

The effect of increasing zinc concentration in NGM media on worms fed with *znuB and znuC* bacteria was a decrease in lifespan. There was no difference in lifespan of worms grown on WT bacteria under the same zinc conditions (p>0.1).

	0 Zn^{2+}	**1µM Zn^{2+}**	**10µM Zn^{2+}**	**100µM Zn^{2+}**
WT	20.4	22.0	21.4	19.9
znuB	22.0	22.2	21.7	20.5
znuC	22.1	23.0	22.0	19.0

Table 1: Summary of *C. elegans* lifespan upon feeding with WT, *znuB*, and *znuC* bacteria grown in NMG media supplemented with different zinc concentrations.

Excluded results of Mn, Fe, and Cu metal analysis

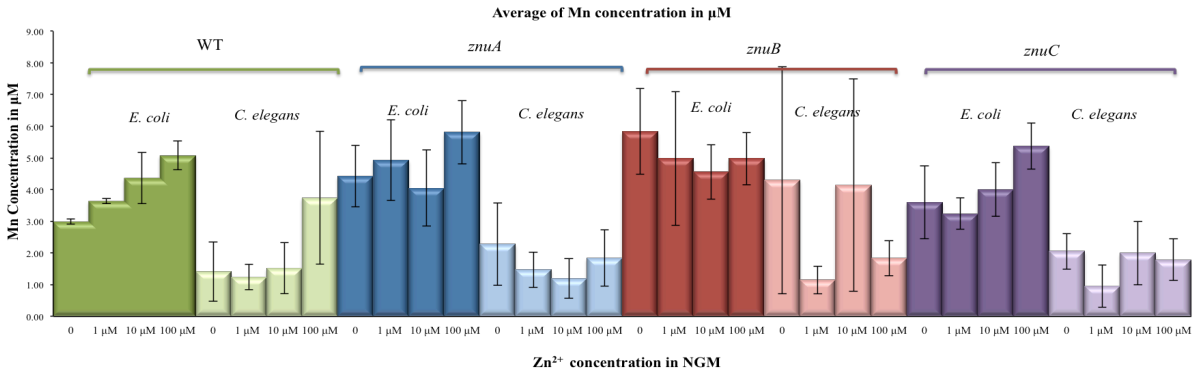

Figure 2: Mn concentration measured in 4 strains of *E. coli* and *C. elegans* fed with the respective bacterial strains, cultured with adding different concentrations of zinc to the NGM media. The experiments were carried out in triplicates. Only WT bacteria showed a gradual increase in Mn concentration as zinc concentration was raised in the NGM ($p<0.02$), and this effect was not observed in *znuABC* mutant strains. As evidenced by the relatively large standard deviation (error bars), there was considerable variation in the measurements of Mn concentration in the 3 mutants bacteria and worms fed with these bacteria. Therefore, despite an overlap between the SD bars from different measurement groups that suggests lack of significant differences in Mn concentration. Data are depicted as mean ±standard deviation (SD).

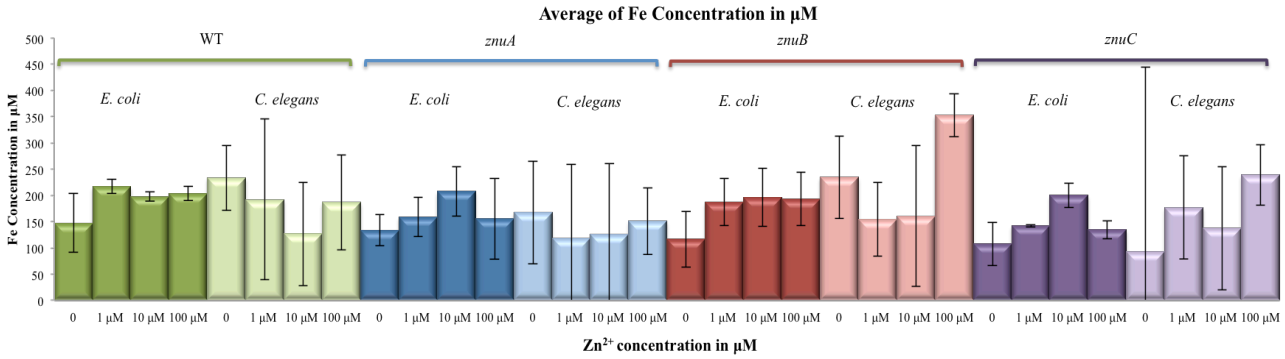

Figure 3: Fe concentration measured in 4 strains of *E. coli* and *C. elegans* fed with the respective bacterial strains, cultured with adding different concentrations of zinc in the NGM media. These experiments were carried out in triplicates. There is no significant difference in Fe concentration between WT bacteria and the 3 mutant strains, and any differences in Fe content between worms fed with different bacterial strains are also not statistically significant ($p>0.1$). Adding different zinc concentration in the NGM media had no effect on Fe concentration in bacteria or woms fed with them. There was considerable variation in the concentration of Fe in the WT and mutant bacterial strains, and worms fed with these bacteria, across the different external zinc concentrations in the NGM media, which again makes the definite conclusions dubious. Data are presented as mean ±standard deviation (SD).

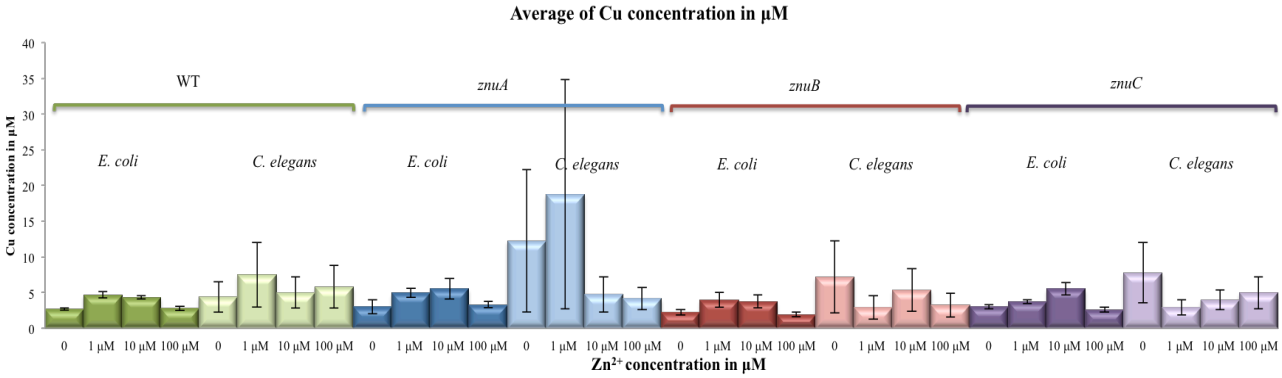

Figure 4: Cu concentration measured in 4 strains of *E. coli* and *C. elegans* fed with the respective bacterial strains, cultured with adding different concentrations of zinc in the NGM media. These experiments were carried out in triplicates. There is no significant difference in Cu concentration between WT bacteria and the 3 mutants. Although worms fed with the mutant bacterial strains showed a trend towards higher Cu content than worms fed with WT bacteria, this difference did not reach statistical significance ($p>0.1$). Supplementing the NGM media with different zinc concentrations had no effect on the Cu concentration, either in bacteria or worms fed with them. As with other metal measurements, there was considerable variability in the results, and therefore despite the apparent lack of significant differences these data need to be interpreted with caution. Data are depicted as mean ±standard deviation (SD).

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