Aus dem Institut für Mikrobiologie und Tierseuchen

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Effects of zinc on protein expression of porcine commensal and pathogenic *Escherichia coli* (*E. coli*)

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Dedicated to my dear parents, sisters and girlfriend!

“I avoid looking forward or backward, and try to keep looking upward!”

- by Charlotte Brontë

"Don't find fault, find a remedy."

- by Henry Ford

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LIST OF ABBREVIATIONS

2D-DIGE	Two dimensional difference gel electrophoresis
2-DE	Two dimensional gel electrophoresis
AAS	Atomic Absorption Spectrometry
APEC	Avian pathogenic *E. coli*
APS	Ammonium persulfate
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate
DAEC	Diffusely adherent *E. coli*
DTE	Dithioerythritol
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
E. coli	*Escherichia coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
fM/cell	Femtomolar per cell
h	Hour
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IMT	Institut für Mikrobiologie und Tierseuchen
InPEC	Intestinal pathogenic *E. coli*
Kb	Kilo base pair
KCl	Potassium chloride

KH_2PO_4	Monopotassium phosphate
LB medium	Luria (Bertani) Broth medium
LB agar	Luria Bertani agar
MALDI-TOF MS/MS	Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectrometry/Mass Spectrometry
Mb	Million base pair
M	Molar
MIC	Minimal inhibitory concentration
MIC_{zinc}	Minimal inhibitory concentration of zinc
mM	Milimolar
MOWSE	**Mo**lecular **W**eight **Se**arch
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NH_3	Ammonia
NMEC	New-born meningitis causing *E. coli*
PBS	Phosphate bufferred Saline
PCR	Polymerase chain reaction
pI	Isoelectronic point
PMF	Peptide mass fingerprint
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SePEC	Septicemia associated *E. coli*
STEC	Shiga toxin-producing *E. coli*
TEMED	N, N, N', N'-tetramethylenediamine
TFA	Trifluoroacetic Acid
UPEC	Uropathogenic *E. coli*
VAG	Virulence associated gene
WGS	Whole genome sequence

TABLES AND FIGURES

CHAPTER I INTRODUCTION

1.1. The importance of zinc

In 1869, zinc was first shown to be essential for the growth of *Aspergillus niger* [1]. The role of zinc in biological function was first recognized and described in 1934 [2]. Since then, it has been demonstrated that zinc is an essential element for all types of life, such as plants, fungi, bacteria, animals and humans [3]. The zinc-containing enzyme, carbonic anhydrase which requires zinc for its enzymatic activity, was described in 1940 [4]. Following this, there was a tremendous increase in knowledge about the important role of zinc in biological, physical and chemical properties, such as ranging from the functions in cellular physiology and identification of zinc-binding proteins, to the structure of zinc metalloenzymes [3, 5-7].

1.1.1. Cellular functions of zinc

As a transition metal, zinc is required for numerous cellular functions, such as DNA synthesis and enzymatic reactions [8]. Zinc is the second most abundant trace metal next to iron and necessary for the survival of all living organisms [6, 9, 10]. The total intracellular zinc concentration in *E. coli* K-12 cultured in minimal medium is estimated to be about 0.2 mM, and in vertebrates it accounts for about 0.003% of body weight [10, 11]. The biological functions of zinc are broadly grouped into three categories: catalytic, structural and regulatory [6]. It has been determined that over 300 enzymes, covering all six classes, require zinc for their functions and thousands of transcription factors possess one or more zinc atoms [12-16]. In *E. coli*, proteomic and bioinformatics studies revealed that there are about 200 proteins containing zinc-binding sites which account for 3-5% of the total proteome [17-19].

The widespread requirement of zinc as cofactor can be associated with its favorable chemical properties [20]. In comparison to other trace metal ions like Cu^{2+} and Fe^{2+}, the zinc ion (Zn^{2+}) does not have the redox potential since from a chemical perspective it contains a filled *d* orbital [21, 22]. Thus, it cannot generate reactive hydroxyl radicals by perturbing the cellular redox reactions. Furthermore, being a stable divalent cation, Zn^{2+} can form complexes with various ligands, such as amino acids, peptides, water, nitrogen and nucleotides. These

complexes are displayed in varied coordination geometries such as distorted tetrahedral coordination polyhedrons or trigonal bipyramidal geometries facilitating the stabilization of the structured protein domains [23]. And, these coordination geometries of structure indicate the possibility of at least three types of zinc-binding sites in enzymes serving these functions: catalytic, co-catalytic and structural stability [7].

Zinc can interact with four specific amino acid residues in particular, sulfur of cysteine (Cys), nitrogen of histidine (His), oxygen of aspartate (Asp) or glutamate (Glu) as well as with one water molecule [24]. In case of catalytic functions, the zinc ion binds to three amino acid residues and mostly one water molecule as the fourth ligand, for example, alkaline phosphatase [25, 26]. The cocatalytic activity is fulfilled by the combination of two or more zinc ions and/or other metal ions coordinated in close proximity such as leucine aminopeptidase [27]. The structural stability of the zinc containing enzyme is often reinforced by zinc binding to Cys forming a tetrahedral coordination that resembles a disulfide bond as in the case of alcohol dehydrogenase [7, 28].

Zinc also binds to transcription factors involved in the regulation of cell division, DNA and RNA synthesis in eukaryotes as well as to proteins associated with DNA repair and oxidative stress response in bacteria [29-34]. These zinc-binding sites in DNA-binding proteins have three distinct motifs: zinc-fingers, twists and clusters, according to structural determination with X-ray crystallographic or Nuclear Magnetic Resonance (NMR) methods [7, 12]. Each motif contains multiple zinc atoms forming different structural characteristics based on various interatomic Zn-Zn distances and/or zinc ligands [7]. The motif of zinc-fingers mostly observed is when each zinc tetragonally binds to two Cys and two His residues forming an α-helical zinc peptide loop that appears repeatedly within DNA-binding sites. For example, the first discovered transcription factor IIIA (TFIIIA) in Xenopus is coordinated as the C_2H_2 zinc finger motif and zinc-finger motifs are also expressed in *E. coli* [7, 35, 36]. In a zinc twist, each zinc is coordinated to four Cys residues and α-helix part of the linking peptide region between two zinc atoms functions as a DNA-recognition site, as in the case of glucocorticoid receptor [12]. The zinc-cluster motif seen in metallothioneins is formed by six Cys residues bound to two zinc atoms, nine Cys residues binding to three zinc atoms or

eleven Cys residues bound to four zinc atoms, which functions as a DNA-binding site, like the GAL4 transcription factor in *E. coli* [7, 12].

1.1.2. Zinc and animal health

Zinc is an essential micronutrient necessary for various physiological functions and is absorbed predominately by the duodenum and jejunum and partly by the stomach. Zinc plays an important role in cell-mediated immune responses by modulating immune functions and anti-inflammatory response [37-42]. It also plays a role in cell signaling, proliferation and differentiation [43]. In addition, zinc acts as an antioxidant and inhibitor of apoptosis, and maintains the integrity of mucosal barriers thereby facilitating resistance to infection [44-47]. A study has revealed that zinc has effects on the healing of skin lesions through enhancement of re-epithelialization [48].

High concentrations of zinc are assumed to have prophylactic and therapeutic functions [49]. It was shown that zinc supplementation can enhance gut health, reduce incidence of diarrhea caused by pathogenic *E. coli* in post-weaning piglets, increase growth performance through enhancement of feed uptake and prevent the increase of intestinal tight junction permeability induced by enterotoxigenic *E. coli* [44, 50-53]. Zinc feeding might also improve mucosal repair and was shown to downregulate the expression of genes associated with inflammation *in vitro* (IPEC J2 cells challenged with enterotoxigenic *E. coli*) and *in vivo* as shown in rat models [39, 53-55]. Supplementation of zinc is probably able to attenuate gastroenteritis due to its antimicrobial effects. It is assumed to enhance enterobacterial diversity, such as coliforms in piglets which in turn might reduce the colonization of pathogenic *E. coli* in the intestine through competition for potential adhesion sites [49, 56, 57]. However, excess zinc might also result in adverse effects in animals such as pica, hypochromic anemia and necrosis of the gastrointestinal tract [58]. Meanwhile, the bioavailability or absorption of copper and iron can be reduced due to competition in binding to metallothioneins responsible for absorption in intestinal cells [56].

On the other hand, zinc deficiency leads to growth retardation in animals due to decreased feed consumption and reduced efficiency of feed utilization, which is often accompanied by

episodes of diarrhea [59, 60]. In addition to that, zinc deficiency results in parakeratosis first described in pigs in 1955, acrodermatitis enteropathica and alopecia in human [61-63]. It also influences the immune response mainly through the decrease in number and maturation of T cells, the reduction of antibody formation, and the impairment of growth and functions of neutrophil and native killer cells [38, 40, 41, 64-68]. Zinc deficiency also contributes to ulceration of intestinal villus and to altering of barrier functions in porcine endothelial cells [69, 70].

1.2. *Escherichia coli*

Escherichia coli (E. coli), first isolated from a healthy infant in 1855, is characterized as a Gram-negative, facultative anaerobic and rod-shaped bacterium that belongs to the family of Enterobacteriaceae [71]. It colonizes the small and large intestine of various hosts such as humans, mammals, fish and birds [72]. *E. coli*, the most abundant and dominant facultative anaerobe of the intestinal microbiota, can colonize the gastrointestinal tract of its host within a few hours after birth [73]. The *E. coli* species is highly individual and dynamic in distribution and its composition varies with host, diet, age and climate [72, 74, 75]. It is also frequently found in the environment as its "secondary habitat", such as soil, sewage and feces [76-79].

E. coli is a versatile bacterium with a high diversity of genome plasticity achieved through horizontal gene transfer [80]. The genome size of *E. coli* is reported to be in the range of 4.6 Mb to 6.2 Mb [81, 82]. Currently, the pangenome of *E. coli* is shown to possess about 16,000 genes in total with about 1,000 genes representing the maximum common genome of the species, while the remaining genes represent the flexible genome [82, 83]. Therefore, the diversity of phenotypes and genotypes results from a combination of many different genes among the 16,000 gene families.

E. coli is grouped as commensal (non-pathogenic) and pathogenic based on manifestations of clinical symptoms and pathology in combination with its virulence traits [84]. The commensal *E. coli* strains are harmless to the host while the pathogenic strains can cause various diseases, such as diarrhea, septicemia, urinary tract infections and meningitis [85]. The pathogenic

strains are broadly classified as intestinal (InPEC) or extraintestinal *E. coli* (ExPEC). Both InPEC and ExPEC display distinct pathotypes based on the specific virulence properties, the mechanisms of disease process, clinical picture and/or host species specificity [86]. As depicted in Figure 1-1, InPEC includes six pathotypes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) [87]. They are mainly capable of causing diarrhea in human and animals which often leads to a large burden of disease and heavy economic losses [73, 88]. ExPEC strains are associated with extraintestinal infections that include: 1. urinary tract infections in humans and animals caused by uropathogenic *E. coli* (UPEC), 2. new-born meningitis due to new-born meningitis causing *E. coli* (NMEC), 3. septicemia caused by septicemia associated *E. coli* (SePEC), and 4. colibacillosis caused by avian pathogenic *E. coli* (APEC) [89].

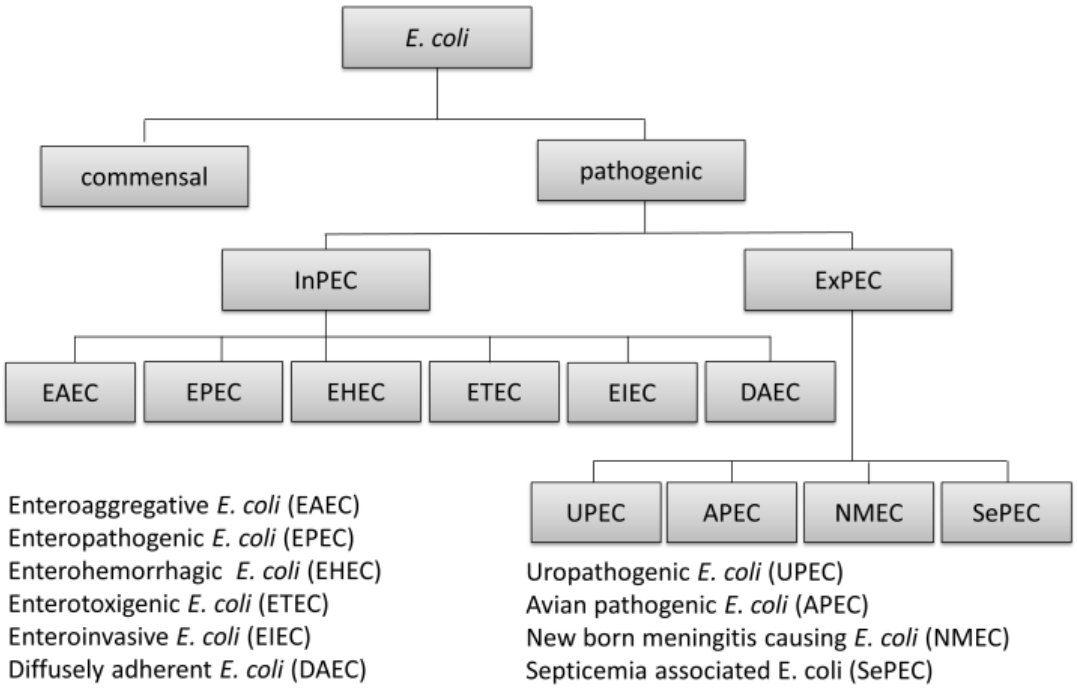


Figure 1-1. Schematic presentation of *E. coli* pathotypes

Phylogenetic analysis using multilocus enzyme electrophoresis (MEE), multilocus sequence typing (MLST) or triplex PCR divides the population of *E. coli* into four main groups (A, B1, B2 and D) on the basis of the *E. coli* reference collection (ECOR), which contains 72 reference strains out of 2600 isolates from natural populations [57, 85, 90]. Most of the

commensal *E. coli* strains belongs to ECOR groups A and B1 lacking most virulence factors, while intestinal pathogenic *E. coli* strains are members of groups A and D with distinctive virulent factor characteristics. In contrast, extra-intestinal pathogenic *E. coli* (ExPEC) strains predominantly derive from group B2 and to a lesser extent from group D [85, 91].

1.2.1. Commensal *E. coli*

Commensal *E. coli* is part of the autochthonous intestinal microbiota out of more than 500 bacterial species within the gastrointestinal tract of human and mammals [80]. It coexists with the host, bringing many benefits such as contributing to the maintenance of microbial and mucosal gut balance as well as homeostasis of the intestinal immune system and playing a role in supporting digestion and vitamin synthesis [72, 73, 92-94]. Furthermore, these commensal bacteria protect, to an extent, against inflammation within the central nervous system and provide probiotic effects by preventing the colonization of intestinal pathogens in humans and animals [95-97]. The initial adhesion of *Salmonella* and EPEC is reduced due to the probiotic activity of commensal *E. coli* [98, 99] .

However, commensal *E. coli* might also cause diseases in immunocompromised hosts or in hosts suffering from gastrointestinal tract diseases such as gut ischemia or intestinal obstruction [100, 101]. Under these conditions bacteria including commensal *E. coli* might breach the barrier of the intestinal epithelium, leading to an increased risk of sepsis or multiple organ failure [101, 102]. In addition, an increasing number of studies show that commensal *E. coli* may act as a reservoir of virulence associated factors for pathogenic *E. coli* and antibiotic resistance genes that can be spread in commensal and pathogenic *E. coli* [99].

1.2.2. Intestinal pathogenic *E. coli* (InPEC)

Intestinal pathogenic *E. coli* (InPEC) strains, also referred to diarrheagenic *E. coli*, mainly colonize the gastrointestinal tract of humans and mammals [87, 103]. They have adapted to cause diarrhea in different hosts via related but distinct mechanisms of pathogenesis and transmission [73]. The specific virulence associated genes of these adapted *E. coli* strains are acquired via horizontal gene transfer: transposons, plasmids, bacteriophages and

pathogenicity islands [104]. The virulence factors encoded by these virulence associated genes are grouped into adhesins, invasins, iron-acquisition factors, serum resistance proteins, toxins and protectins [73]. The combination of these factors mediates the characteristics of different pathotypes and causes various clinical symptoms, such as watery and hemorrhagic diarrhea, as well as colitis [87].

Among the six well-described pathotypes of InPEC, EPEC associated with diarrhea in children and animals possesses the locus of enterocyte effacement (LEE) but lacks *stx,* encoding Shiga toxin [105]. Two subgroups, typical and atypical EPEC (see Chapter 1.2.3), are known and can be divided according to the presence or absence of the EPEC adherence factor (EAF) plasmid [106]. EHEC is a human pathogen that causes hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), which are attributed to the Shiga toxin type 1 and 2 or both, encoded by *stx1* and *stx2* genes respectively [107, 108]. EAEC, which is mostly responsible for persistent diarrhea in human, exhibits the presence of the *set* gene encoding Shigella enterotoxin-1, *sen* gene encoding Shigella enterotoxin-2 or *astA* gene encoding heat stable toxin 1 [109]. DAEC is particularly associated with persistent diarrhea in children over one-year old in developing countries, and is characterized by a unique adhering pattern, diffuse adherence, as observed in cases of cultured epithelial cells [110]. The full adherence activity is attributed to two genes, *aah* and *aidA* [111]. ETEC possesses heat-labile enterotoxins (LTs) encoded by the *elt* gene or heat-stable enterotoxins (STs) encoded by the *est* gene and can cause watery diarrhea in children in developing countries and travelers, as well as in swine and bovine [112]. EIEC is mainly associated with watery diarrhea, invasive inflammatory colitis and dysentery in human, which is mediated by a plasmid-borne type III secretion system that secretes multiple proteins, such as IpaA, IpaB and IpaC encoded by different subtypes of the *ipa* gene [87].

1.2.3. Enteropathogenic *E. coli* (EPEC)

EPEC is a leading cause of human diarrhea, especially among infants in developing countries, and is also a cause of diarrhea occurring in animals [113, 114]. EPEC infections result in a characteristic lesion, known as attaching and effacing (A/E), which is also observed in EHEC

and *Citrobacter rodentium* [115-117]. The pathogenesis of A/E lesion in histopathology is that the bacteria intimately attach to intestinal epithelium and then form a pedestal-like structure, accompanied by aggregation of actin filaments beneath the attached bacteria and effacement of local microvilli of the intestine [117, 118].

The virulence factors involved in the formation of A/E lesion are encoded by genes located in a 35-kb chromosomal pathogenicity island, named locus of enterocyte effacement (LEE) [119]. The LEE contains the genes encoding: 1) an outer membrane adhesin termed intimin responsible for the intimate adherence between bacteria and enterocytes by Tir-intimin interactions; 2) the translocated intimin receptor Tir; 3) a type III secretion system, and secreted proteins involved in cell signaling [119-121].

In 1995, EPEC, characterized by the presence of the LEE pathogenicity island and the absence of *stx* genes, was classified into two subgroups, typical EPEC (EPEC) and atypical EPEC (aEPEC) [114, 121]. The EPEC strains carry the EPEC adherence factor (EAF) plasmid encoding the type IV bundle-forming pilus (BFP), which mediates the formation of a characteristic localized adherence (LA) in cell culture, represented by compact microcolonies [122]. The aEPEC lacks the EAF plasmid and differs in adherence pattern showing either a diffuse adherence (DA) pattern mediated by the Afa adhesion, a localized adherence-like (LAL) pattern mediated mainly by intimin, or an aggregative adherence (AA) pattern mediated by aggregative adhesion [106, 121, 123]. Therefore, EPEC strains with *eae*+ *bfp*+ *stx*- genetic background are categorized as EPEC with LA adherence pattern. On the other hand, aEPEC strains that are *eae*+ *bfp*- *stx*- have three distinct adherence patterns: LAL, DA and AA [124].

Comparatively, EPEC is frequently isolated from human diarrhea cases, while aEPEC is shown to be isolated from various animals, such as pigs, dogs, sheep and chickens with diarrhea [86, 122, 125, 126].

1.2.4. Extraintestinal pathogenic *E. coli* (ExPEC)

ExPEC strains possess various virulence factors that enable them to invade and cause diseases

in niches outside of the gastrointestinal tract, including the blood, the central nervous system, the urinary tract and the respiratory system in humans and animals [127, 128]. These strains pose a great threat to public health worldwide with high morbidity and mortality associated with substantial economic costs [129]. ExPEC rarely contributes to diarrhea, although it can asymptomatically colonize the gastrointestinal tract of humans and animals [127, 130, 131]. Hence, the gut of mammals or birds acts as a potential reservoir of ExPEC [132-134]. It was demonstrated that a high prevalence of virulence-associated genes belonging to ExPEC is also expressed in commensal *E. coli* isolated from the intestine of clinically healthy swine [134, 135].

ExPEC strains exhibit a large genome diversity represented by a broad range of encoded virulence associated factors, including diverse adhesins, invasins, iron-acquisition factors, polysaccharide capsules, serum resistance proteins, toxins and protectins, which are frequently encoded by mobile elements such as plasmids and pathogenicity islands [100, 136]. ExPEC is defined by the presence of two or more of the following virulence markers determined by multiplex PCR: *papA* (encoding P fimbriae structural subunit) and/or *papC* (P fimbriae assembly), *afa/dra* (encoding Dr-antigen-binding adhesins), *sfa/foc* (encoding S and F1C fimbriae subunits), *iutA* (encoding aerobactin receptor and *kpsMT II* (encoding group 2 capsular polysaccharide units), or by identification of o454-nlpD (novel lipoprotein) genomic region using PCR amplification and sequencing [127, 136, 137].

1.3. Zinc and *E. coli*

1.3.1. Zinc hemostasis in *E. coli*

Zinc is an essential metal ion for numerous processes in *E. coli* growth. However, excess zinc is deleterious. Thus, zinc homeostasis is tightly regulated upon the mechanisms of the influx and efflux systems to avoid balance disorders and cellular damage [8].

The amount of intracellular zinc needed for the optimal growth of *E. coli* is termed as zinc quota proposed by T. O' Halloran [11]. The zinc quota of *E. coli* cultured in LB medium is estimated to be 4 x 10^5 atoms/cell [11]. Whereas, the minimal quota of *E. coli* cells grown in a

metal-deleted medium is about 2 x 10^5 atoms/cell, which corresponds to 0.2 mM in total calculated as zinc concentration per cell, about ~2000 times higher than the total zinc concentration (1 x 10^{-7} M) measured in the minimal medium [11]. In yeast and mammalian cells, the zinc quotas were estimated to be about 10^7 and 10^8 atoms of zinc per cell respectively, using atomic absorption spectrometry (AAS) [138]. Although the values calculated for *E. coli*, yeast and mammalian cells vary over three orders of magnitude, when adjusted for cell volume of all cell types the total cellular zinc concentrations range from 0.1 mM to 0.5 mM [138]. However, based on the previous measurements, the internal free zinc concentration in *E. coli* is extremely low with considerable variability and is proposed to be in the femtomolar to picomolar range [11, 139, 140].

In *E. coli* there are at least four known zinc membrane transport systems regulating bacterial zinc homeostasis through sensing fluctuations of zinc, as shown in Figure 1-2 [8]. Under zinc deficient conditions, zinc uptake is mediated by two major influx pumps: ZnuABC, a high affinity ABC-type zinc uptake system when growing in an environment with very low zinc availability, and ZupT, a low-affinity transporter functioning under conditions of moderate zinc availability [141, 142]. The ZnuABC whose synthesis is tightly regulated by the *zur* gene, consists of three components: ZnuA, a periplasmic binding protein; ZnuB, a membrane spanning protein; and ZnuC, an ATPase [142, 143]. ZupT, responsible for zinc uptake in *E. coli,* is a member of the ZIP (for ZRT/IRT-like protein) family transporters, which are reportedly responsible for transporting iron, zinc, cadmium or manganese in eukaryotes [144-146]. Zinc detoxification is mainly accomplished by two efflux pumps, P_{1B}-type ATPase ZntA and cation diffusion facilitators (CDF) ZitB [147, 148]. ZntA synthesis is regulated by ZntR, a member of the MerR family of regulators, while the expression of *zitB* is activated directly by zinc [147, 149]. In addition to ZntA and ZitB, it was shown that the BaeSR (bacterial adaptive response) regulon that controls the expression of transporters such as *acrD*, *mdtC* and *mdtD* also participates in defending against zinc toxicity in *E. coli* [150].

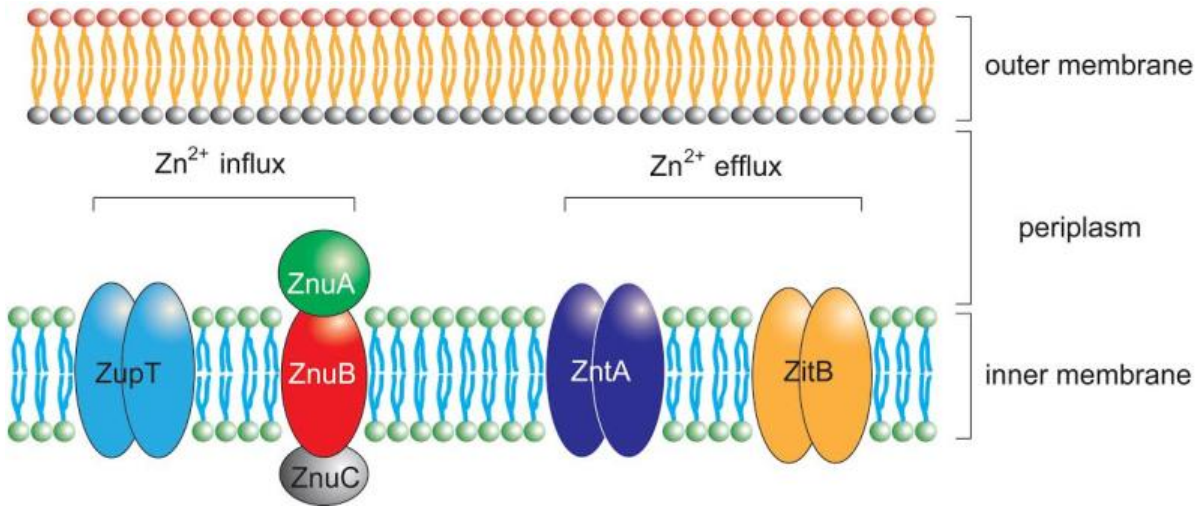


Figure 1-2. The main zinc transporting systems in *E. coli*

1.3.2. Effects of zinc on virulence traits of *E. coli*

Due to its antibacterial effects, zinc has been widely used to prevent or reduce diarrhea in children in developing countries and in livestock [151, 152]. Because of this, numerous recent studies have focused on the mechanisms by which zinc exerts its effects on the microbiota in the intestine and the host or *in vitro* [153-155].

Results have shown that the micronutrient zinc has the ability to inhibit the adherence to epithelium, and reduce biofilm formation and virulence factor expression in EAEC [156]. The oral supplementation of zinc has been shown to reduce the rate of translocation of pathogenic bacteria from the small intestine to the ileal mesenteric lymph node [157].

In EPEC, zinc causes a decrease in type III-secretion system-dependent secretion of proteins, like EspA and EspB, and inhibits the expression of genes located on the LEE4 and LEE5 operons of the LEE pathogenicity island as well as the formation of bundle-forming pilus, thereby reducing EPEC adherence to cultured cells and attenuating the virulence of this pathogen [155, 158]. Similarly, it was previously demonstrated that a micromolar concentration of zinc results in increased membrane permeability and suppressed virulence genes expression of EPEC, which is mediated by RpoE stress response pathway [158, 159].

In EHEC, zinc decreases the amount of secreted EspA (*E. coli* secreted protein) and inhibits

EHEC-associated histological damages of intestinal epithelial cells [154]. In addition, the expression of Shiga toxin is downregulated both at the genetic and protein level [154]. It is suggested that the inhibitory effects of zinc on Shiga toxin expression might be mediated by a reduced expression of *recA*, which is a reliable marker of the SOS stress response pathway that is a powerful regulator of EHEC Shiga toxin production. Thus, the inhibition of SOS response is proposed to be one mechanism by which zinc reduces the EHEC infection [153].

1.3.3. Proteomic analysis of the effects of zinc on *E. coli*

To the best of our knowledge, only two studies have been carried out concerning the adaptive response of *E. coli* to external zinc at the proteomic level. Both studies focused on the laboratory strain *E. coli* K-12 strain using 2D gels [160, 161]. The response of *E. coli* exposed to 0.2 mM $ZnSO_4$ is reported to be time-dependent, since the identified differentially expressed proteins, mainly involved in transport, protein translation and glycolysis, vary between 30 min and 4 h of incubation time [161]. The second study showed that most identified proteins influenced by 0.25 mM Zn^{2+} are related to cellular metabolisms, such as glycolytic and tricarboxylic acid (TCA) associated enzymes and membrane transporters [160].

1.4. Antibiotic resistance mechanisms

The high prevalence of antibiotic resistance in a wide range of bacteria is a serious global problem, since it becomes an increasing threat to public health associated with increased morbidity and mortality, as well as huge economic costs [162, 163]. Bacteria utilize a variety of mechanisms to resist antimicrobial agents. The molecular mechanisms of resistance are grouped into intrinsic resistance and acquired resistance [164]. An intrinsic antibiotic resistance is naturally encoded and expressed by all strains of a bacterial species and is characterized by the presence of low-affinity antibiotic targets or the general absence of these targets, and by low permeability or presence of effective efflux mechanisms, all of which contribute to resistance against a particular drug or antibiotic class [165]. As one example, *E. coli* has an inherent resistance to vancomycin [164]. The acquired resistance may be a result of the mutation of one or more chromosomal genes involved in normal physiological

processes and cellular structures, or of horizontal gene transfer (transformation, transduction or conjugation), or a combination of these two mechanisms [164, 166]. The acquisition of exogenous genes occurs via the uptake of mobile genetic elements including plasmids or free DNA (conjugation or transformation), integrons and bacteriophages (transduction) and transposons (conjugation), as shown in Figure 1-3 [167]. These mobile resistance determinants can be disseminated between strains of the same bacterial species or even between different species [168]. Through genetic exchange the bacteria are able to gain abilities to be resistant against multiple classes of antibiotics, thus leading to a broad emergence of multi-resistance in bacteria [162, 169].

The main types of biochemical mechanisms involved in antimicrobial resistance are shown in Figure 1-3. They include enzymatic inactivation or modification, target site alternations or overexpression, alternation of metabolic pathway and reduced intracellular drug accumulation [168, 170, 171]. The enzymatic inactivation or modification of the antimicrobial substance is mainly achieved by three enzymes: β-lactamases, chloramphenicol acetyltransferases and aminoglycoside-modifying enzymes, mostly encoded by resistance genes, which can abolish antimicrobial activities by transferring adenyl or acetyl groups to antimicrobials or hydrolyze antimicrobial agents [170, 172, 173]. The cellular target sites of antimicrobials are altered in different ways, such as peptidoglycan structure alteration, protein or DNA synthesis interference, for example, methylation of 23S ribosomal RNA results in the combined resistance to chloramphenicol, florfenicol and clindamycin [174, 175]. The acquired genes are probably responsible for an alternative metabolic pathway that bypasses antimicrobial substances' action [168]. The reduced intracellular drug accumulation is due to increased efflux or reduced outer membrane permeability [176, 177]. In Gram-negative bacteria, antibiotics such as β-lactams, tetracycline and fluoroquinolones can penetrate the outer membrane representing the permeability barrier by the three mechanisms: porins, bilayer or self-promoted uptake, e.g. through OmpF and OmpC in *E. coli* [164, 176, 178-180]. A large number of organisms, such as *E. coli* and *P. aeruginosa*, obtain the antibiotic resistance through loss, down-regulation or functional change of porins (e.g. OmpF, NmpC and OmpC) acting as an entry of drug molecules [176-178]. Therefore, altered permeability is one

mechanism involved in antibiotic resistance.

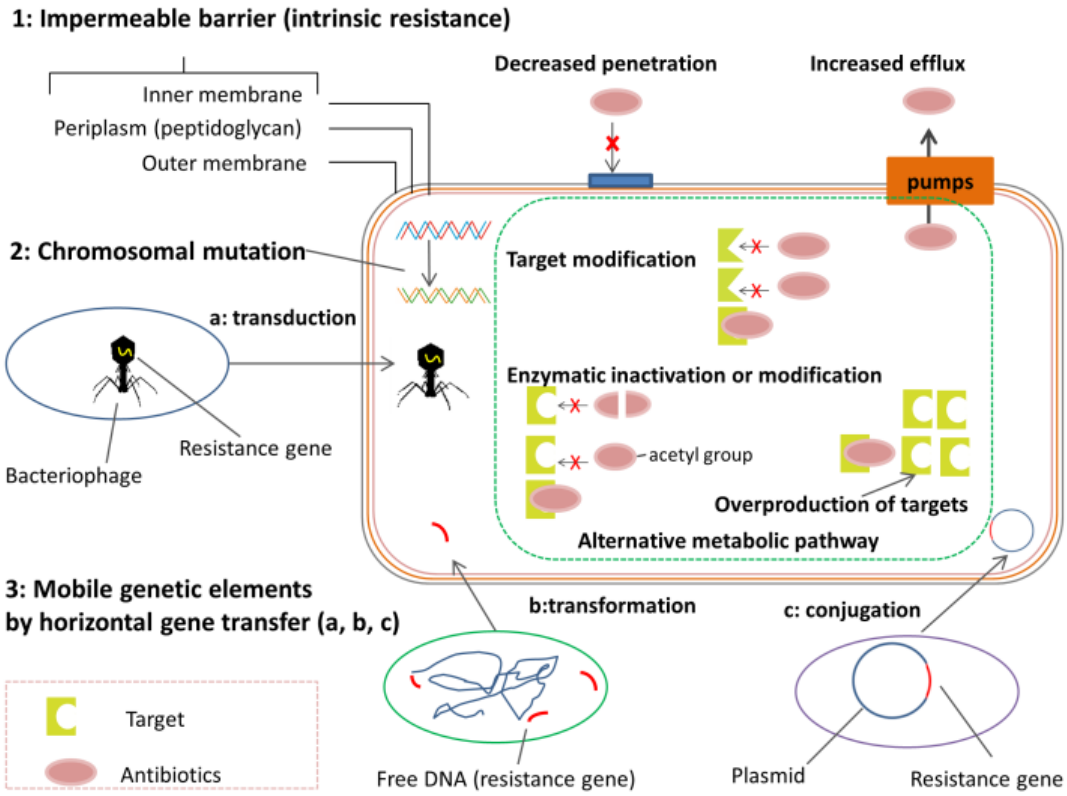


Figure 1-3. Schematic description of antibiotic resistance mechanisms.

1.5. Aims of the project

Since 2006, in-feed antimicrobial substances used as growth promoter for food-producing animals have been prohibited in the European Union (EU). The main intention was to reduce the prevalence of antibiotic resistant bacteria [181]. In the EU, addition of zinc (in low dosage) in the feeding diets of farmed animals is to meet the requirements of physiological activities and consequently to improve the growth performance (Regulation (EC) No 1831/2003). In some EU countries such as the UK and Denmark but not in Germany, it has been approved that high dosage of zinc oxide (2500 ppm) is used as a prophylactic and therapeutic treatment to prevent the diarrhea caused by pathogenic *E. coli*.

Intestinal *E. coli* is a predominant bacterial species containing commensal and pathogenic bacterial strains with high relevance for animals. Thus, in-feed zinc actually effects both populations. Regarding the effects on commensal *E. coli*, which makes up a great part of gut

microbiota, earlier studies revealed that zinc supplementation in excess of the nutritional requirements of the host not only increases the *E. coli* diversity, but also results in the development of resistance to zinc and a concomitant cross-resistance to antibiotics [57, 182]. However, the mechanism for antimicrobial resistance driven by zinc exposure remains unclear. Moreover, estimates of the intracellular amount of zinc in wild-type *E. coli* strains are lacking. Zinc supplementation has a protective effect, providing resistance against diseases such as diarrhea caused by pathogenic *E. coli*. This might be due to zinc's suppressing the virulence genes expression of these pathogens such as EPEC and EHEC [154, 155], while the molecular mechanisms involved are still elusive. In addition, there is no available knowledge concerning how the wild-type *E. coli* responds to zinc stress at the proteomic level. So far only *E. coli* K-12 has been analyzed, on the other hand, lab strains are highly different in gene content and physiology compared to wild-type strains [160, 161, 183].

Based on this background, the following aims were chosen for the present study:

I: Characterization of two porcine intestinal *E. coli* strains (commensal and pathogenic).

II: Evaluation of zinc sensitivity and the influence of zinc on growth rates.

III: Determination of intracellular zinc concentration in commensal *E. coli in vitro* over time.

IV: Quantitative proteomic analysis to evaluate the influence of zinc on the protein expression of porcine intestinal *E. coli* strains.

CHAPTER II MATERIAL AND METHODS

2.1. Material

2.1.1. Bacterial strains

E. coli IMT29408

The strain was isolated during a previously performed piglet feeding trial [57, 184] from the intestine of a 33 day-old piglet. In previous characterization, it was found to represent a Pulsed-field gel-electrophoresis (PFGE) clone that was present in 50% of all trial animals, in both the animals fed a control diet (50 ppm) and the high zinc feeding group (2500 ppm). The strain has been deposited in the culture collection of the Institute of Microbiology and Epizootics (Freie Universitaet-Berlin, Germany).

E. coli IMT8073

IMT8073 was isolated in 2003 from the intestine of a four-day-old piglet suffering from diarrhea. Previous characterization identified this strain as a member of the atypical enteropathogenic *E. coli* (aEPEC) that harbors the locus of enterocyte effacement (LEE) and causes attaching and effacing (A/E) lesions, but lacks the adherence factor (EAF) plasmid. The whole genome of IMT8073 was sequenced previously [86] and the hemolytic strain is deposited at the culture collection of Institute of Microbiology and Epizootics (Freie Universitaet-Berlin, Germany).

In the present study, both strains were comprehensively analyzed using both phenotypic and genotypic methods, as described below (Chapter 2.2.1).

E. coli DH5α (K-12)

E. coli K-12 is a laboratory reference strain since it lacks the specific virulence characteristics and has the poor capability to colonize the intestine in human and animals [81]. It grows readily on common laboratory culture media and it is demonstrated that it is a valuable tool for microbial physiology and genetics research. In our study, we used this strain as a comparison with wild-type strains. This is also from the culture collection of Institute of

Microbiology and Epizootics (Freie Universitaet-Berlin, Germany).

2.1.2. Media, buffers and solutions

Growth medium

Luria Bertani (LB) Medium (25.0 g/l)

NaCl	5.0 g /l
Peptone (casein)	10.0 g /l
Yeast extract	5.0 g /l
pH	6.0/7.0

Autoclaved at 121ºC, 15 min

pH 6.0 was used for bioreactor culture while pH 7.0 was utilized to perform MIC of zinc and antibiotic resistance test.

Luria Bertani Agar (40.0 g/l)

NaCl	5.0 g /l
Peptone (casein)	10.0 g /l
Yeast extract	5.0 g /l
pH	7.0
Agar	15 g/l

Autoclaved at 121ºC, 15 min

Solutions for zinc sensitivity and intracellular zinc measurement test

Zinc stock solution

$ZnCl_2$, 373.13 mM measured by atomic absorption spectrometry

NaCl 0.9%

Sodium chloride	9.0 g
MiliQ-water	1000.0 ml

Autoclaved at 121ºC, 15 min

Phosphate Buffered Saline (PBS, 10x)

NaCl	80.0 g/l
KCl	2.0 g/l
Na_2HPO4	14.4 g/l

KH_2PO4 2.4 g/l
Dissolved in 800.0 ml MiliQ-water and pH adjusted to 7.4 and volume filled up to 1.0 l.
Autoclaved at 121ºC, 15 min.

PBS (1x)

Diluted from 10 x PBS in MiliQ-water and then autoclaved at 121ºC, 15 min.

MiliQ-water

Autoclaved at 121ºC, 15 min

1.85% HCl solution

50.0 ml 37% HCl diluted with 950.0 ml ultrapure water

2.5% HCl solution

Diluted from 25% HCl stock solution with MiliQ-water

7.5% NH_3 solution

Diluted from 15% NH_3 stock solution with MiliQ-water

Solutions for protein extraction

HEPES lysis buffer (pH 7.4, 20.0 mM)

HEPES	0.0953 g
Triton X-100	200.0 µl
10% Glycerol	2.0 ml
0.5% protease inhibitor	100.0 µl
EDTA	1.0 mM

Filled up to 20.0 ml with MiliQ-water

Solutions and Buffers for 2D-DIGE

Ice Acetone (100%)

Collected 250.0 ml from the stock and stored at -18°C for protein precipitation

0.5 M Tris/HCl pH 6.6 (500.0 ml)

Tris-base	30.285g
MiliQ-water	400.0 ml

Vortex by magnet and adjusted pH to 6.6 with HCl
Filled up to 500.0 ml with MiliQ-water and filtered by paper and store at 4°C

1.5 M Tris/HCl pH 8.8 (500.0 ml)

Tris-base 90.86 g
MiliQ-water 400.0 ml
Vortex by magnet and adjusted pH to 8.8 with HCl
Filled up to 500.0 ml with MiliQ-water and filtered by paper and stored at 4°C

Acrylamide (30.8% T; 1.0 l)

Acrylamide 300.0 g
Bisacrylamide 8.0 g
MiliQ-water 800.0 ml
Dissolved and vortex by using magnetic fish for 1 h
Filled up to 1000.0 ml with MiliQ-water and filtered by paper and stored at 4°C

10% Sodium dodecyl sulfate (SDS)

SDS 10.0 g
MiliQ-water 100.0 ml
Vortex by using magnetic fish

10% Ammonium persulfate (APS)

APS 1.0 g
MiliQ-water 10.0 ml
Vortex by using magnetic fish

SDS-PAGE sample loading buffer (100.0 ml)

MiliQ-water 50.0 ml
0.5 M Tris/HCl Buffer (pH 6.8) 12.5 ml
Glycerol 10.0 ml
10% SDS 20.0 ml
Mercaptoethanol 5.0 ml
Bromophenol Blue 0.5-1.0 g

Equilibration buffer (0.05 M trichloroethylene HCl pH 8.8, 250.0 ml)

Glycerol 94.5 g
Urea 90.09 g

SDS 10.0 g
0.5M Tris-HCl, pH8.8 25.0 ml
Bromine phenol blue 0.5-1.0 g
Vortex by magnet and adjusted pH with HCL to 8.8, filled up to 250.0 ml with MiliQ-water, then stored at -20°C

Equilibration solution I

Equilibration Buffer 5.0 ml
DTE 100.0 mg
Prepared just before use

Equilibration solution 2

Equilibration Buffer 5.0 ml
Iododacetamide 125.0 mg
Prepared just before use

Electrophoresis buffer (10x buffer- dilute to 1x just before use)

Tris-base 30.3 g
Glycine 144.0 g
Dissolved in about 600.0 ml MiliQ-water and vortex
Filled up to 1000.0 ml with MiliQ-water
Added carefully 10.0 g of SDS and vortex by magnet for 30 min (filtration not required)

1% Agarose

1x SDS electrophoresis buffer 100.0 ml
Agarose 1.0 g
Heated up to 90°C in microwave for 2-3 min.

Coomassie staining solution

Prepare 1.0 l of Coomassie staining solution as follows:
MiliQ-water 100.0 ml
Ortho phosphoric acid 85% 100.0 ml
Ammonium sulphate 100.0 g
100% methanol 100.0 ml
Coomassie Blue G-250 1.2 g
Shaken with magnet, then added MiliQ-water to fill up to 1000.0 ml and stored at room temperature for 6 months.

DIGE labeling buffer (pH 9.0)

Urea	42.0 g
Thio urea	15.4 g
4% CHAPS	4.0 g
30 mM Tris	0.364 g

Dissolved in 100.0 ml MiliQ-water and adjusted pH to 9.0

Dye quenching buffer (10.0 mM)

Lysine	1.0 g
MiliQ-water	100.0 ml

Solutions and buffers for trypsin digestion

Gel washing solution (200.0 mM Ammonium bicarbonate)

Ammonium carbonate	1.5812 g
HPLC water	100.0 ml

Desaturation solution (200.0 mM Ammonium bicarbonate with 50% acetonitrile)

Ammonium carbonate	1.5812 g
Acetonitrile	50.0 ml
HPLC water	50.0 ml

Digesting solution (20.0 mM Ammonium bicarbonate with 5% acetonitrile)

Ammonium carbonate	0.015812 g
Acetonitrile	5.0 ml
HPLC water	95.0 ml

Trypsin enzyme solution

Modified sequencing grade trypsin	100.0 µg
100% acetic acid	3.0 µg

Filled up to 1000.0 µl with HPLC water
Aliquot it into 20.0 µl and store at - 20°C

TFA solution (10 ml)

33% Acetonitrile	3.3 ml
TFA (0.1%)	10.0 µl

HPLC water 6.7 ml

HCCA matrix solution

α-Cyano-4-hydroxycinnamic acid 10-20.0 mg
TFA solution 1.0 ml
Vortex for 1 min and stood in dark for 10 min, then centrifuged for 10 sec using mini centrifuge

2.1.3. Sources for chemicals, reagents, media, consumables and equipment

Company	Name
Analytik Jena, Jena, Germany	Atomic absorption spectrometry (ContrAA 700)
AnaeroGen, Oxoid, Hants, UK	AnaeroGen sachet
AppliChem, Darmstadt, Germany	Dithioerythritol (DTE)
	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
	Mercaptoethanol
Binder, Tuttlingen, Germany	Incubator for bacteria plates
Bio-Rad, Munich, Germany	Bovine Serum Albumin (BSA)
	Cellophane sheets
	GelAir Dryer
	Quick Start Bradford
Brucker, Bremen, Germany	MALDI-TOF MS Ultraflex II TOF/TOF
	MALDI-TOF peptide calibration standard
	MTP 384 target plate
	Peptide Calibration Standard II
	α-Cyano-4-hydroxycinnamic acid
Carl Roth, Karlsruhe, Germany	Acetone
	15% Ammonia solution (NH_3)
	Ethanol
	Ethylenediaminetetraacetic acid (EDTA)
	Glycerol
	Glycine
	25% Hydrochloric acid (HCl)
	Monopotassium phosphate (KH_2PO4)
	Luria Bertani Agar, powder

	Luria (Bertani) Broth Medium, powder
	Disodium phosphate (Na_2HPO4)
	Potassium chloride (KCl)
	Sodium chloride (NaCl)
	Tris base
	Triton X-100
Eppendorf, Wesseling-Berzdorf, Germany	Eppendorf Biophotometer plus
	Eppendorf Centrifuge 5415R
	Eppendorf Centrifuge 5415D
	Multichannel pipet, 100 µl, 300 µl
	Pipettes (10 µl,50 µl,100 µl,200 µl, 1 ml)
	Thermomixer Pro (Cellmedia)
GE Healthcare, Freiburg, Germany	Cy-dyes DIGE Fluor (Minimal dye)
	Destreak Rehydration solution
	Drystrip Cover Fluid
	Electrophoresis Power Supply-ESP301
	Electrophoresis system
	Ettan DALT Power supply & control unit
	Ettan DALT Twelve System
	Ettan IPGphor 3 isoelectric focusing unit
	Iodoacetamide
	IPG buffer
	IPGphor strip holder cleaning solution
GE Healthcare, Uppsala, Sweden	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propan esulfonate (CHAPS)
	Ettan DIGE Image Scanner
Gesellschaft fur für Labortechnik, Burgwedel, Germany	Shaking Incubators
Hielscher, Teltow, Germany	UP100H ultrasonic processor
IKA Labortechnik, Staufen, Germany	Agitator
	Magnetic stirrer
	MS2 Minishaker
	Vortex
Knick, Berlin, Germany	pH/ ion meter

National Labnet Co., Edison, USA	Mini Centrifuge (C-1200)
Merck MilliPore, Darmstadt, Germany	MiliQ-water machine
	Zinc Standard solution (1 g/l)
Merck, Darmstadt, Germany	Ammonium Persulfate (APS)
	Acetic acid
	Acrylamide
	Agarose
	Bisacrylamide
	Coomassie Brilliant Blue R-250
	Methanol
	Ortho phosphoric acid
	Trifluoroacetic acid
Merck, Hohenbrunn, Germany	N, N, N', N'-tetramethylenediamine (TEMED)
	Thiourea
Merck, Schuchardt, Germany	2-Propanol
Nuaire, Plymouth, USA	Laminar flow cabinet
Oxoid, Wesel, Germany	Columbia agar with sheep blood PLUS
PerkinElmer life and analytical sciences, USA	Quality Control Standard 21 (100 µg/ml) for AAS
Promega, Mannheim, Germany	Trypsin enzyme
Sarstedt, Nümbrecht, Germany	Sterile filters 0.2 µm
	96-well blank plate
Sartorius, Göttingen, Germany	Bioreactor (Biostat B plus)
	Bromophenol Blue
	Centrifuge and angle rotor 19776-H
SERVA, Heidelberg, Germany	Weighing Balance
Sigma, Osterode am Harz, Germany	Sodium Dodecyl Sulfate (SDS)
Sigma-Aldrich, Steinheim, Germany	Acetonitrile
	Ammonium carbonate
	Ammonium sulfate
	HPLC water
	L-Lysine monohydrochloride
	Protease inhibitor cocktail
Systec,Wettenberg, Germany	Autoclave
Theodor Karow, Berlin, Germany	Water bath

Thermo Scientific,Erlangen, Germnay	Prestained Protein Marker
	Sensititre inoculator plate holder
	Sensititre SensiTouch
Uniequip, Martinsried, Germany	Vacuum centrifuge UniVapo 100H
VWR, Darmstadt, Germany	Urea
Xylem WTW, Weinheim, Germany	Colony counter

2.1.4. Software

Company	Software Name
Analytik Jena, Jena, Germany	Aspec CS 1.5.4.0
Brucker,Bremen, Germany	Flexcontrol 3.4
	BioTools 3.2
DECODON GmbH, Greifswald, Germany	Delta 2D software Version 4.4
GE Healthcare, Freiburg, Germany	IamgeQuantTL
Lucent Technologies, New Jersey, USA	R Version 3.2.0

2.2. Methods

2.2.1. Genotypic characterization

Databases for the automatic screening of whole genome sequence (WGS) data have been developed at the Institute of Microbiology and Epizootics (IMT). These databases contain reference sequences for *E. coli* virulence associated genes (n=253) and antibiotic resistance genes (n=45), respectively.

Screening of the WGS of both IMT29408 and IMT8073 was performed using an in-house pipeline based on the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (conducted by Torsten Semmler and Flavia Dematheis). A gene is considered as be present if the sequence similarity is > 75% and coverage is > 90%.

2.2.2. Zinc sensitivity test

In order to examine zinc sensitivity of IMT29408, IMT8073 and K-12, a modified MIC assay developed in our institute was performed (guided by Carmen Bednorz and Astrid Bethe). In

brief, 150 µl of 64 mM zinc chloride (1 ml zinc stock ($ZnCl_2$, 373.13 mM) added into 4.8 ml MiliQ-water) was transferred into the first column of 96-well blank plate and then a 2-fold serial dilution was performed with 150 µl MiliQ-water, which created incremental concentrations (0, 0.0313, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 mM) of zinc (Table. 2-1). The plates coated with variant zinc concentrations were dried under laminar air-flow. *E. coli* strains IMT29408, IMT8073 and K-12 were streaked on sheep blood agar plates and incubated overnight at 37°C. Single colonies were then selected and inoculated into 5.0 ml of 0.9 % NaCl solution to reach McFarland Standard of 0.5 measured by Sensititre inoculator plate holder. The inoculum was prepared by adding 50.0 µl of the above bacterial suspension into 11.0 ml of Luria Broth (LB) medium. Following which, 150.0 µl of inoculum of K-12 and IMT29408 or IMT8073 was added to each well accordingly. The plates were incubated for 24 h at 37°C in an anaerobic condition, which was created by sealed box with AnaeroGen sachet. Evaluation of the experiment was performed optically using Sensititre SensiTouch. In line with the MIC determination for antimicrobial substances, the lowest concentration of zinc inhibiting visible growth was determined as MIC_{zinc}.

Table 2-1. Layout of 96-well plate for the determination of MIC_{zinc}.

	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
B	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015
C	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015
D	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015
E	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015
F	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015
G	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015
H	32.0	16.0	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015

Row A: 150µl sterile MiliQ-H_2O/well added and dried under laminar air flow, as growth control; Row B-H: The final zinc concentration of each well (unit: mM) incubated with bacteria.

2.2.3. Bioreactor culture

To mimic the anaerobic conditions present in the intestine and to investigate the influence of

zinc (1.0 mM $ZnCl_2$) on *E. coli*, the experiments were performed using a bioreactor (37°C, pH 6.0 with 200 rpm stirring) and guided by Carmen Bednorz. LB medium (25.0 g/l, pH 6.0) serves as a control while $ZnCl_2$ was added to LB medium to a final concentration of 1.0 mM for the exposure study. The LB media (500.0 ml) with or without 1.0 mM $ZnCl_2$ was taken in to the bioreactor, autoclaved and subjected to overnight stabilization of pH, temperature and atmosphere. The anaerobic environment was created by consecutively flowing nitrogen (N_2) and the pH was maintained at 6.0 with 7.5 % NH_3 and 2.5% HCl automatically. The pre-cultures was performed in 50.0 ml LB medium by inoculation with single colony and incubated overnight at 37°C with 200 rpm shaking in an aerobic environment. After measuring the cell density (OD_{600}) of pre-cultures by Biophotometer, the required volume was inoculated in the bioreactor to reach OD_{600}=0.1.

2.2.4. Influence of zinc on growth kinetics

Growth curves of *E. coli* strains in the presence or absence of 1.0 mM $ZnCl_2$ were determined using a bioreactor mentioned above. Aliquots of 1.0 ml were drawn at every hour initially between 0-7 h to assess the growth in terms of colony-forming units (CFU) per milliliter (termed CFU/ml) and OD at 600 nm (termed OD_{600}). To determine CFU/ml, 100.0 µl of bacterial suspension was serially diluted 10-fold with sterile PBS and three dilutions were plated twice on the LB agar plates. The value of OD_{600} was measured using Biophotometer. The experiment was independently carried out in triplicates.

2.2.5. Intracellular zinc content measurement

Bacteria were cultured using the bioreactor as described above and aliquots were collected every hour (0 h to 10 h, Table 2-2). Different volumes were taken at different time points to reach the limit for quantification in Atomic Absorption Spectrometry (AAS). The bacterial cells were harvested by centrifugation at 2,600 x *g*, 4°C for 10 min. The pellets of bacteria were washed successively with 1x PBS and MiliQ-water and centrifuged at 2,600 x *g*, and 4°C for 10 min. The cell pellets were then resuspended with 1.0 ml sterile MiliQ-water and boiled at 95°C, 60 rpm shaking for 10 min. Subsequently, the suspension was incubated on ice for 10 min and then centrifuged at 10,397 x *g* for 10 min. Finally, the supernatant was

collected for intracellular zinc measurement. In parallel, the CFU/ml was counted at each hour point by 10-fold dilution with sterile PBS; three dilutions were chosen and plated in duplicates on LB agar plates.

Table 2-2. Details on sample collection for intracellular zinc content measurement.

Time point	Volume taken (ml)	Sample source
0	10	from pre-culture
1	100	from bioreactor
2	25	
3	10	
4	10	
5	10	
6	10	
7	10	
8	10	
9	10	
10	10	

Intracellular zinc concentration was measured using AAS. In brief, four different zinc concentrations (0.05 mg/l, 0.1 mg/l, 0.15 mg/l and 0.2 mg/l) were prepared by diluting zinc standard solution (l.0 g/l) and used for constructing a calibration standard curve using Aspec (CS 1.5.4.0) software. The calibration standard curve was evaluated by determining the known zinc concentration of Quality Control Standard solution (100 µg/ml). After calibration of AAS, each sample was diluted with 1.85% HCl solution. The dilution was adjusted according to the concentration of each sample while the total sample volume should exceed 1.5 ml for measurement in AAS. Then, the samples were centrifuged at 7200 x *g*, 20°C for 3 min to remove any impurities. The supernatant of each sample was transferred to the 25.0 ml polypropylene tube and measured automatically for three times, the mean of all three measurements was computed as the final concentration. The intracellular zinc content per cell, interpreted as atoms/cell, was determined by this equation: (Total cellular zinc concentration x Volume (1.0 ml) x N_A)/(Ar(Zn) x Total number of colony-forming units (CFUs), Ar (Zn): 65.38 g/mol; N_A: Avogadro Constant = 6.02 x 10^{23} mol^{-1}). This experiment was supervised by

Astrid Bethe and Jayaseelan Murugaiyan, and the ASS measurement (machine operation) was carried out by Louisa Thies and Anett Kriesten.

2.2.6. Zinc exposure and protein extraction

Zinc chloride (1 mM) exposure to *E. coli* IMT29408 and IMT8073 was carried out in a bioreactor as described above. Aliquots of 20.0 ml bacterial culture were harvested at 2 h and 5 h. The whole cell protein extraction was carried out as described [185]. In brief, the bacterial suspension was centrifuged at 2,600 x *g*, 4°C for 5 min. The supernatant was discarded and the pellets were washed twice with 1.0 ml PBS, centrifuged at 2,600 x *g*, 4°C for 5 min. After discarding the supernatant, the pellets were resuspended with 250.0 µl HEPES buffer (20.0 mM, pH 7.4), incubated on ice for 10 min and lysed by sonication (duty cycle: 1.0, amplitude: 100%, 100 W, 30 kHz) for 55 sec on ice. Subsequently, the samples were centrifuged at 11,290 x *g*, 4 °C for 5 min. The supernatant was collected and stored at -20°C until further analysis. The protein concentration was determined using modified Bradford method and confirmed by taking 10 µg protein of each sample using small 12% SDS-PAGE followed as shown in Table 2-3. The protocols of this part as well as below parts (2.2.7, 2.2.8, 2.2.10) were from Institute of Animal Hygiene and Environmental Health and guided by Jayaseelan Murugaiyan.

Table 2-3. Protocol for casting SDS-PAGE gels.

Composition	**4% stacking gel**	**12% separation gel**
Total volume (ml)	5.0	10.0
MiliQ-water (ml)	3.05	3.5
0.5 M Tris/HCl pH 6.6 (ml)	1.25	
1.5 M Tris/HCl pH 8.8 (ml)		2.5
30.8% Acrylamide (ml)	0.65	4.0
10% SDS (µl)	50.0	100
10% APS (µl)	37.5	75.0
TEMED (µl)	7.5	7.5

2.2.7. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)

2D-DIGE was performed as recommended by the manufacturer (GE Healthcare, Freiburg, Germany) and as described [185]. Per sample, 250.0 µg proteins were precipitated with acetone (five-fold of volume of each sample) for 10 min at -20°C and centrifuged at 7,900 x *g*, 0°C for 10 min. The supernatant was discarded and the pellets were dried at room temperature to remove the traces of acetone. The dried pellets were resuspended with 30.0 µl of labeling buffer and vortexed vigorously. As an internal standard, one third of all samples was pooled and labeled with fluorescent cyanine Cy2 dye (200.0 pmol/100.0 µg protein). The remaining two thirds of every sample was labeled either with Cy3 or Cy5 (200.0 pmol/100.0 µg protein for each dye) in such a way to avoid color quenching. The labeling reaction was carried out in the dark for 30 min on ice and terminated with 1.0 µl of 10.0 mM lysine for 5 min. Following labeling, samples labeled with Cy3, Cy5 and internal standard (Cy2) were pooled. Prior to proceeding towards isoelectronic focusing steps, 0.2 µl of each pooled sample was separated on a 10 x 10 cm SDS-PAGE gel (protocol given in Table 2-3). The gels were scanned and the labelling efficiency was evaluated using the recommended software. After the evaluation of labeling, 450.0 µl of DeStreak rehydration solution supplemented with 0.5% IPG-buffer 3-10 NL was added to each sample. Then these samples were centrifuged at 11,290 x *g*, 20°C for 30 min and the supernatant was added to IPG strip (pH 3-10 NL, 24 cm) and allowed for overnight rehydration. Isoelectric focusing was carried out at 20°C using Ettan IPGphor 3 isoelectric focusing unit (GE Healthcare) for 24 h as recommended by the manufacturer (Table 2-4). The strips were then reduced and alkylated with equilibration buffer supplemented with 2% DTE and 2.5% iodoacetamide for 15 min with shaking at room temperature, respectively. The strips were washed with MiliQ-water and placed on the top of SDS-PAGE gel (12.5% acrylamide, 0.65 cm x 20 cm x 25 cm) and sealed with 1% agar (<60°C). The second dimensional separations were performed using Ettan DALTtwelve electrophoresis system connected to Ettan DALTtwelve system power supply and control unit at 22°C with power supply of 1 W/gel. The electrophoresis was stopped before the blue dye reached the bottom of gel.

Gels were immediately scanned using Ettan DIGE Image Scanner and the following

excitation/emission filters (Cy2:488 nm/520 nm; Cy3: 532 nm/580 nm; Cy5: 633 nm/670 nm) were chosen. The gels were transferred to DIGE imager and the scanning area of each gel was defined in Ettan DIGE imager software. The pixel size was set to 100.0 μm and the images were saved in DIGE File Naming Format. The exposure time was adjusted to a value of intensity (pixel) with that the most intense spot reaches or close to detection saturation by ImageQuantTL software. After scanning, all the gels were stained with Coomassie Brilliant Blue R-250 [186] overnight at room temperature with shaking, destained with MiliQ-water and then dried between cellophane for long-term storage.

The detailed labeling scheme of IMT29408 and IMT8073 were given in Tables 2-5 and 2-6. In summary, a total of 12 gels (6 gels for each time point) were carried out for each strain to meet standards of biological and technical replicates. Dye swapping (Cy3 and Cy5) was performed for each sample to compensate any difference in dye-protein binding of different Cy dyes.

Table 2-4. Isoelectronic focusing conditions.

	Voltage (V)	**Time (h)**
Step 1: Step and Hold	500	1.0
Step 2: Gradient	1000	7.0
Step 3: Gradient	8000	3.0
Step 4: Step and Hold	8000	5.5
Step 5: Gradient	10000	3.0
Step 6: Step and Hold	10000	4.0
		Total :23.5 h

“Step and hold”: set the voltage for the new step and hold this voltage constant for the step duration; “Gradient”: the voltage increases in increments (linearly with respect to time) from the value set for the previous step to the value set for this step.

Table 2-5. Samples labelling scheme of IMT29408.

Sample(ID)	Source	Cy3	Cy5	Gel No.	Strip No.	File name
2 h samples						
IMT29408_2h/1	zinc treatment	YES		1	50265	01_50265_zn2_ctrl2_20130816
IMT29408_2h/A	control		YES			
IMT29408_2h/2	zinc treatment	YES		2	50266	02_50266_zn2_ctrl2_20130816
IMT29408_2h/B	control		YES			
IMT29408_2h/3	zinc treatment	YES		3	50267	03_50267_zn2_ctrl2_20130816
IMT29408_2h/C	control		YES			
IMT29408_2h/1	zinc treatment		YES	4	50269	04_50269_zn2_ctrl2_20130816
IMT29408_2h/C	control	YES				
2IMT29408_h/2	zinc treatment		YES	5	50268	05_50268_ctrl2_zn2_20130816
IMT29408_2h/A	control	YES				
IMT29408_2h/3	zinc treatment		YES	6	50270	06_50270_ctrl2_zn2_20130816
IMT29408_2h/B	control	YES				
5 h samples						
IMT29408_5h/1	zinc treatment	YES		7	50271	07_50271_zn5_ctrl5_20130816
IMT29408_5h/A	control		YES			
IMT29408_5h/2	zinc treatment	YES		8	49846	08_49846_zn5_ctrl5_20130816
IMT29408_5h/B	control		YES			
IMT29408_5h/3	zinc treatment	YES		9	49847	09_49847_zn5_ctrl5_20130816
IMT29408_5h/C	control		YES			
IMT29408_5h/1	zinc treatment		YES	10	49848	10_49848_ctrl5_zn5_20130816
IMT29408_5h/B	control	YES				
IMT29408_5h/2	zinc treatment		YES	11	49849	11_49849_ctrl5_zn5_20130816
IMT29408_5h/C	control	YES				
IMT29408_5h/3	zinc treatment		YES	12	49850	12_49850_ctrl5_zn5_20130814
IMT29408_5h/A	control	YES				

Note: to distinguish three independent replicates between zinc treatment and control, the replicates in zinc treatment named in figure: 1, 2 and 3; replicates in control named in character: A, B and C.

Table 2-6. Samples labelling scheme of IMT8073.

Sample(ID)	Source	Cy3	Cy5	Gel No.	Strip No.	File name
2 h samples						
IMT8073_2h/1	zinc treatment		YES	1	49853	01_49853_ctrl2_zinc2_20150115
IMT8073_2h/A	control	YES				
IMT8073_2h/2	zinc treatment		YES	2	49854	02_49854_ctrl2_zinc2_20150115
IMT8073_2h/B	control	YES				
IMT8073_2h/3	zinc treatment		YES	3	50257	03_50257_ctrl2_zinc2_20150115
IMT8073_2h/C	control	YES				
IMT8073_2h/3	zinc treatment	YES		4	49855	04_49855_zinc2_ctrl2_20150115
IMT8073_2h/A	control		YES			
IMT8073_2h/1	zinc treatment	YES		5	49856	05_49856_zinc2_ctrl2_20150115
IMT8073_2h/B	control		YES			
IMT8073_2h/2	zinc treatment	YES		6	49857	06_49857_zinc2_ctrl2_20150115
IMT8073_2h/C	control		YES			
5 h samples						
IMT8073_5h/1	zinc treatment	YES		7	49858	07_49858_zinc5_ctrl5_20150115
IMT8073_5h/C	control		YES			
IMT8073_5h/1	zinc treatment		YES	8	49859	08_49859_ctrl5_zinc5_20150115
IMT8073_5h/A	control	YES				
IMT8073_5h/2	zinc treatment		YES	9	49860	09_49860_ctrl5_zinc5_20150115
IMT8073_5h/B	control	YES				
IMT8073_5h/3	zinc treatment		YES	10	49861	10_49861_ctrl5_zinc5_20150115
IMT8073_5h/C	control	YES				
IMT8073_5h/3	zinc treatment	YES		11	49862	11_49862_zinc5_ctrl5_20150115
IMT8073_5h/B	control		YES			
IMT8073_5h/2	zinc treatment	YES		12	50258	12_50258_zinc5_ctrl5_20150115
IMT8073_5h/A	control		YES			

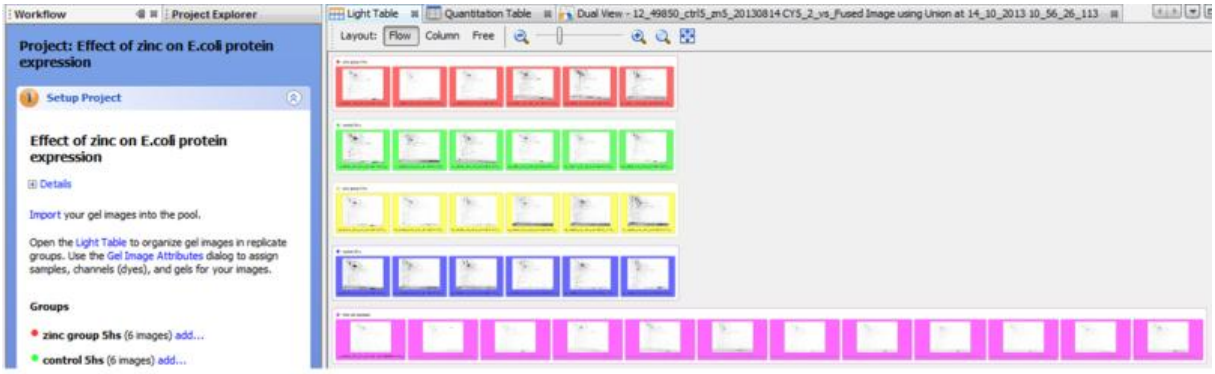
Note: to distinguish three independent replicates between zinc treatment and control, the replicates in zinc treatment named in figure: 1, 2 and 3; replicates in control named in character: A, B and C.

2.2.8. Quantitative gel analysis

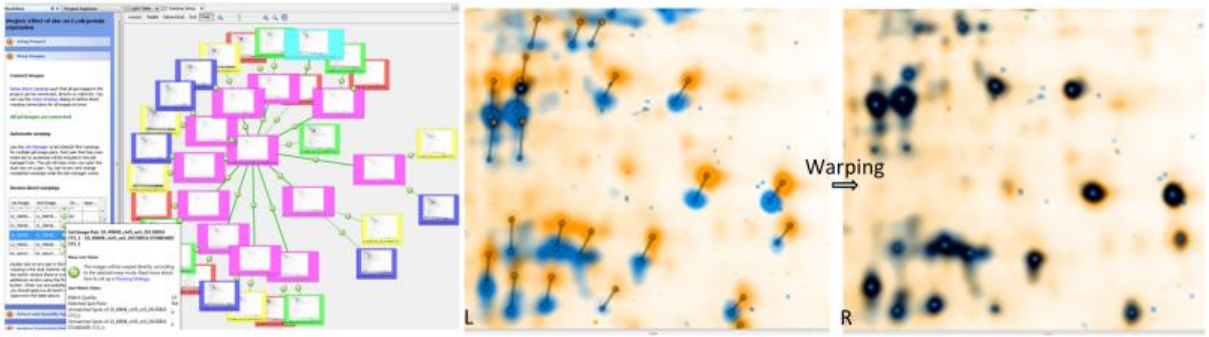
Quantitative analysis was performed using Delta 2D software (Version 4.4) according to the recommendations of the manufacturer (www.decodon.com) [187]. Firstly, a project was created in Delta2D and all the gel images (Cy2, Cy3 and Cy5) were imported into the project and assigned as groups in accordance with experimental setup. All the internal standard images (labeled with Cy2) across the whole experiment were configured to one group. In-gel standard warping strategy was used to align the internal standard gels. Within the same gel, the other two images (labeled with Cy3 and Cy5) do not need any warping because they are co-migrated automatically by warping internal standard images. Image warping makes all the spots of the same protein have the same position across the gels so as to eliminate the distortions of the gels, which are achieved by aligning spots on one image with corresponding spots on master image under a dual channel image window. Image warping can be performed first automatically by Smartvectors that can generate matched vectors connecting identical spot between the gel images. Then, these matched vectors were edited by manual adjustment, e.g. creating, deleting and changing.

After warping, all the images were fused together to generate a fusion image (artificial master gel image) which contains all the spots of gel images in the project. The spots were detected automatically and edited manually on the master gel. Each spot was then given unique ID number on the fused image, thereby creating a consensus spot pattern for the whole project. The consensus spot pattern was transferred from the fusion image to all the gel images where the spots were quantified automatically according to pixel intensity. Each spot volume was normalized on the total protein amount by intensity on each gel (excluding the biggest spots accounting for ~ 5% of total intensity from the normalization). Relative volumes of the spots were determined in comparison to intensity of the same spots in the internal standard channel on each gel. Mean relative volumes of identical spots on the triplicate gels were calculated and divided by the mean relative volume of the corresponding spots in the controls, yielding the expression ratio [187]. The protein spots with more than 1.5-fold change (student's *t*-test, $p<0.05$) were considered as differentially expressed. Then all these data were exported in Excel or PowerPoint. Some typical operation steps are indicated in Figure 2-1.

Figure 2-1. Selective illustration of operation steps in Delta2D

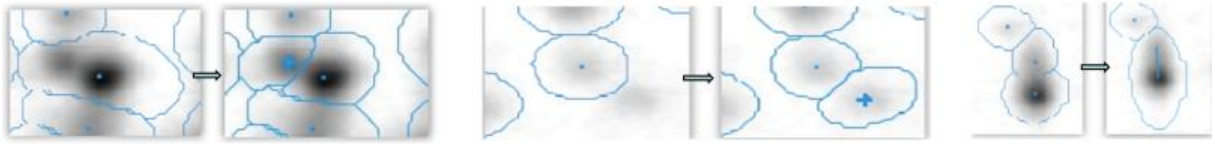


1. Gel images imported to Delta2D



2. In gel standard warping strategy (distribution tree)

3. Before warping: L; after warping: R

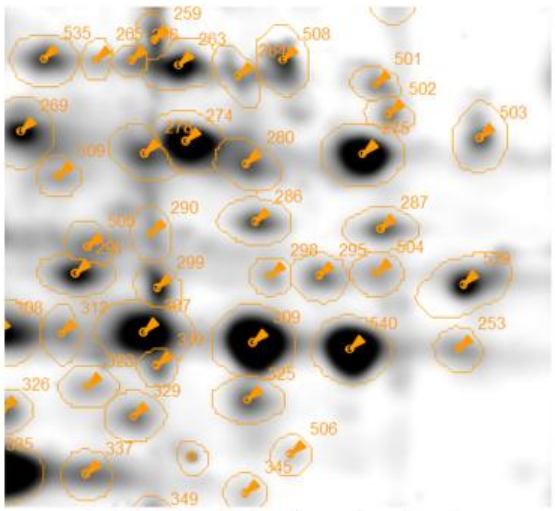


L: Splitting a spot

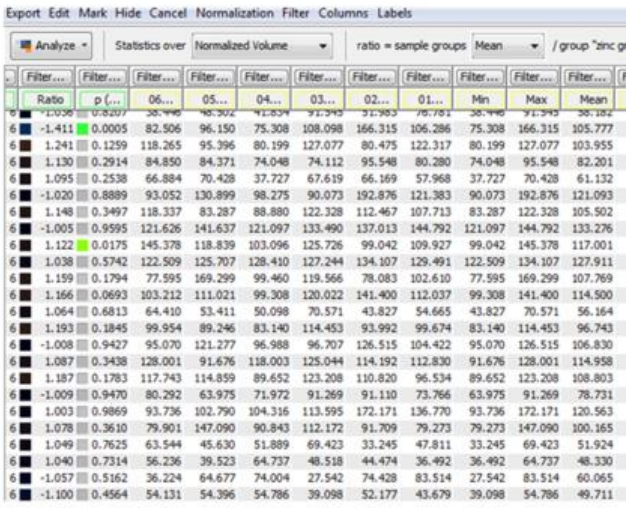
M: Adding a new spot

R: Joining two spots to one spot

4. Spot editing



5. Naming ID number of each spot



6. Quantitation Table for each spot across all of the gels

Note: L:Left; M:Middle, R: Right; The images were cropped from data of our project using Delta2D software (www.decodon.com).

2.2.9. Trypsin digestion

The differentially expressed spots were excised from Coomassie Blue stained gels and in-gel digested with trypsin was carried out as described [188]. Bovine serum albumin on the gel was chosen as a standard for evaluation of trypsin efficiency. The gels pieces were washed with 200.0 µl MiliQ-water and then destained by 30 min incubation with shaking at room temperature with each of the following steps subsequently: 1. two hundred µl of 200.0 mM ammonium bicarbonate; 2. two hundred µl of 200.0 mM ammonium bicarbonate in 50% acetonitrile 3. two hundred µl of 20.0 mM ammonium bicarbonate in 5% acetonitrile. Subsequently, the spots were shrunken with 50.0 µl acetonitrile for 5 min and dried using vacuum centrifuge. To each gel piece, 25.0 µl of trypsin solution (0.1 µg/ µl) was added and incubated overnight at 37°C. The resulting peptides were extracted with 25.0 µl acetonitrile. This extraction was repeated three times, and the extracts were pooled and dried completely using vacuum centrifuge.

2.2.10. Protein identification

The dried peptide extracts were then reconstituted with 5.0 µl of 3% acetonitrile in 0.1% trifluoroacetic acid. After 10 sec centrifugation using mini centrifuge, 1.0 µl of this extract solution was mixed with 1.0 µl of matrix (1.5% α-Cyano-4-hydroxycinnamic acid in 33% acetonitrile in 0.1% trifluoroacetic acid) and spotted 1 µl of the mixture on a ground steel MTP 384 target plate.

The MALDI-TOF MS/MS measurement was carried out using Ultraflex II TOF/TOF instrument system by Jayaseelan Murugaiyan and Christoph Weise [185]. The external calibration standard (peptide calibration standard II) supplied by the manufacturer was used to calibrate the machine in the mass range of 1000 and 3500 Da. The peptide calibration standard II consists of seven standard peptides listed in Table 2-7. The MALDI-TOF/TOF peptide mass fingerprint (PMF) ion spectra were acquired in positive refection mode where the accelerating voltage was set to 25.0 kV. Each spectrum was generated by accumulating data from 300 laser shots. The MALDI-TOF/TOF generated ion spectra were recorded in laser-induced dissociation mode (LID) at an initial accelerating voltage of 26.3 kV in the

primary ion source and a re-acceleration potential of 13.6 kV in the LIFT cell.

Following measurement, a database search was carried out using BioTools 3.2 software by combination of PMF and MS/MS spectra through MASCOT (www.matrixscience.com) against all entries of NCBInr (GenBank) databases with subsequent parameters: trypsin digestion up to one missed cleavage, fixed modifications (carbamidomethyl cysteine), variable modifications (methionine oxidation), mass tol. MS (100 ppm), MS/MS tolerance (±0.8 Da) and peptide charge (1+). The protein identification was considered as valid when the MOWSE (**MO**lecular **W**eight **SE**arch) score (-10*log (P), where P is the absolute probability, and protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits) was greater than the significant value (student‘s *t*-test, $p<0.05$), in combination with at least 2 peptides sequences matched to the MS ions [188]. In addition, the pI and MW of the identified proteins were physically matched to the positions of the respective spots on the gel. Figure 2-2 depicts the PMF and MS/MS ion spectra generated through MALDI-TOF MS/MS measurement of BSA sample. The PMF and MS/MS were combined together using the BioTools software and the mass list was used to pattern matched with that of the database information (NCBI). The BSA identification was determined by assigning an absolute probability value to a MOWSE score (www.ionsource.com), and all the protein hits were ranked according to protein scores in Figure. 2-3.

Table 2-7. Compounds of peptide calibration standard II

Peptide	[M+H]+ Mono isotopic	[M+H]+ Average
Angiotensin II	1046.5418	1047.19
Substance P	1347.7354	1348.64
Bombesin	1619.8223	1620.86
ACTH clip 1-17	2093.0862	2094.43
ACTH clip 18-39	2465.1983	2466.68
Somatostatin 28	3147.4710	3149.57

This data from Brucker (Part No.:222570, www.bruker.com).

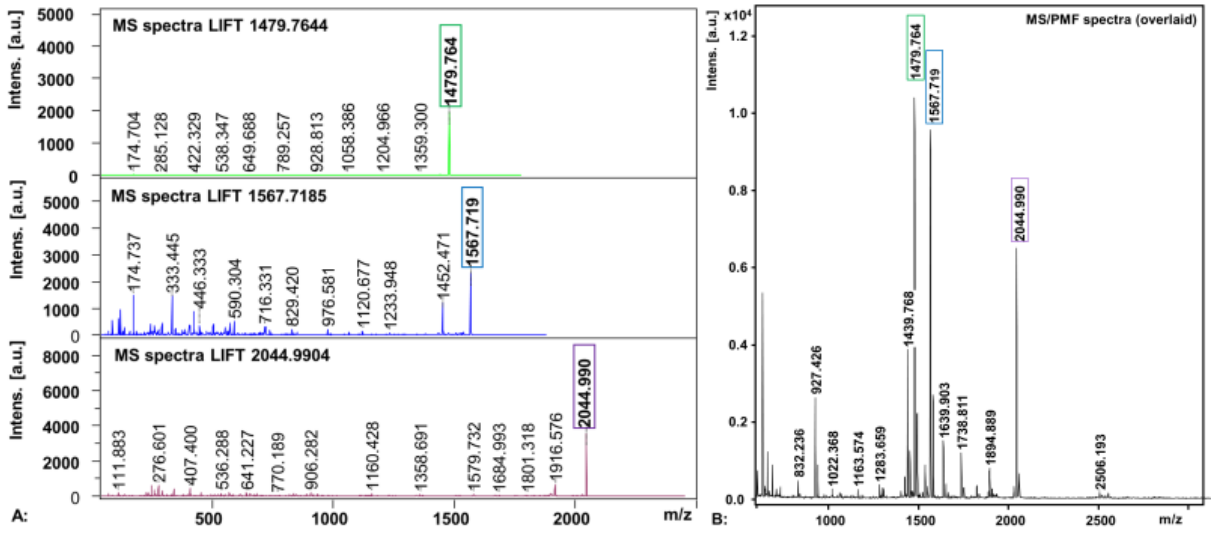


Figure 2-2. Representative composition of individual MS spectra (A) of BSA and combination of these spectra (B).

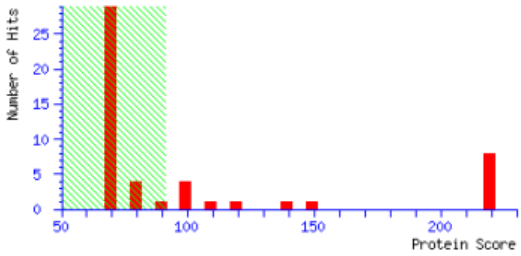
The individual MS spectra of BSA from MALDI-TOF (www.ionsource.com) and the combination of these spectra by Bio-tools that used for database search.

2.2.11. Bioinformatic analysis

In order to gain further insights into the relevant interaction network and biological functions of differentially regulated proteins, CLC Genomics Workbench with BLAST2GO plugin was used for Gene Ontology (GO) classification searched in BLAST2GO database (www.blast2go.com) as the manufacturer guided (performed by Torsten Semmler) [189]. There are four steps composed of gene ontology classification. Firstly, the sequences of aimed genes that encode the differentially expressed proteins identified were imported in Workbench and blasted against NCBI database. Then GO terms were mapped on the basis of the blast results using annotation files from GO consortium, thus retrieving GO terms associated to the hits obtained after a BLAST search. After mapping, the resulting GO terms from the GO pool were selected and assigned to query sequences. These sequences were annotated by applying an annotation rule (AR) though setting up parameters to find the most specific annotations with a certain level of reliability. Based on this, these annotations were visualized on the main application table represented by enrichment of biological processes and validated by removing redundant GOs from the dataset. This analysis was combined with the searching in Ecocyc *E. coli* database (http://ecocyc.org/).

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 91 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Protein Summary Report

Format As: Protein Summary (deprecated) — Help
Significance threshold p< 0.05 — Max. number of hits 20
Preferred taxonomy: All entries

Index

	Accession	Mass	Score	Description
1.	gi|367460260	68416	219	Chain A, Crystal Structure Of Bovine Serum Albumin
2.	gi|594045062	71177	216	PREDICTED: serum albumin [Bubalus bubalis]
3.	gi|1351907	71244	216	RecName: Full=Serum albumin; AltName: Full=BSA; AltName: Allergen=Bos d 6; Flags: Precursor [Bos
4.	gi|555958369	71288	216	PREDICTED: serum albumin [Bos mutus]
5.	gi|30794280	71274	216	serum albumin precursor [Bos taurus]
6.	gi|742111094	71259	216	PREDICTED: serum albumin [Bison bison bison]
7.	gi|74267962	71186	216	ALB protein [Bos taurus]
8.	gi|154425704	71244	215	ALB protein [Bos taurus]
9.	gi|76445989	55487	148	serum albumin [Bos indicus]
10.	gi|229552	68083	142	albumin
11.	gi|597718071	68102	116	serum albumin, partial [Cervus nippon]
12.	gi|556777536	71127	109	PREDICTED: serum albumin [Pantholops hodgsonii]
13.	gi|193085052	68266	104	albumin precursor [Capra hircus]
14.	gi|685425595	68281	104	Chain A, Crystal Structure Of Ovine Serum Albumin
15.	gi|548470611	71124	104	PREDICTED: serum albumin [Capra hircus]
16.	gi|57164373	71139	104	serum albumin precursor [Ovis aries]
17.	gi|795078906	48080	86	PREDICTED: G2/mitotic-specific cyclin-B [Vollenhovia emeryi]
18.	gi|640790511	71191	78	PREDICTED: serum albumin [Tarsius syrichta]
19.	gi|700270399	8235	76	hypothetical protein NZ45_18830 [Clostridium botulinum]
20.	gi|358331857	63032	76	eukaryotic translation initiation factor 3 subunit D [Clonorchis sinensis]

Results List

1. gi|367460260 **Mass:** 68416 **Score:** 219 **Expect:** 8.5e-15 **Matches:** 9
Chain A, Crystal Structure Of Bovine Serum Albumin

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Ions	Peptide
927.4262	926.4189	926.4861	-72.54	137	143	0	---	K.YLYEIAR.R
1163.5740	1162.5667	1162.6234	-48.76	42	51	0	---	K.LVNELTEFAK.T
1283.6586	1282.6513	1282.7034	-40.59	337	347	0	---	R.HPEYAVSVLLR.L
1439.7678	1438.7605	1438.8045	-30.56	336	347	1	35	R.RHPEYAVSVLLR.L
1479.7644	1478.7572	1478.7881	-20.95	397	409	0	---	K.LGEYGFQNALIVR.Y
1567.7186	1566.7113	1566.7354	-15.42	323	335	0	76	K.DAFLGSFLYEYSR.R
1639.9028	1638.8955	1638.9305	-21.34	413	427	1	5	R.KVPQVSTPTLVEVSR.S
1888.8811	1887.8738	1887.9195	-24.20	145	159	0	---	R.HPYFYAPELLYYANK.Y
2044.9904	2043.9831	2044.0206	-18.35	144	159	1	53	R.RHPYFYAPELLYYANK.Y

No match to: 832.2356, 941.4401, 1022.3684, 1297.6704, 1307.6104, 1403.4784, 1424.9513, 1453.7793, 1461.7233, 1493.7698, 1
1894.8887, 1908.9020, 2030.1303, 2058.9978, 2506.1927, 2555.1189

Figure 2-3. MASCOT search result of BSA.

The mascot score histogram showed the number of protein hits distributed on the basis of protein scores. The protein summary program displayed the parameters of data searching. Index showed the list of protein hits including accession number in the database, protein score, mass and description. The resulting list provides the full details about the peptides of BSA.

CHAPTER III RESULTS

3.1. Genotypic characterization

3.1.1. Virulence associated genes (VAGs) screening

IMT29408 possesses a total of 18 out of the 253 virulence associated genes (VAGs) included in the screening database (supplemental table 1). These genes were categorized as coding for adhesins, toxins/hemolysins, serum resistance proteins/protectins and miscellaneous genes as shown in Table 3-1. Among a total of eighteen detected genes, most of these genes, e.g. *ompT* encoding outer membrane protease VII (OmpT) and *fimC* encoding subunit of Type 1 fimbriae, are known to be widely distributed among both pathogenic and non-pathogenic *E. coli* strains. Only three of them (*aidA*, *iss* and *csgA*) were reported to be specifically associated with increased virulence in InPEC or ExPEC.

In contrast, the genome of IMT8073 carried 56 VAGs out of the 253 targets shown in Table 3-1. It harbors a marker virulence gene of EPEC, *eae* encoding intimin, located on the LEE pathogenicity island, but lacks other typical virulence genes like *bfp*, *elt, est, aggR, ipa, stx1* or *stx2* (Supplemental table 1). Other genes carried on the LEE pathogenicity island (e.g. *espA, espD, espG, espH, escC, escD,* and *escF*) were part of the VAGs detected, as well as non-LEE encoded genes such as *nleA* and *nleE*. As already mentioned in chapter 2, the whole genome of this strain has been published previously [86].

Table 3-1. Virulence associated genes present in respective IMT29408 and IMT8073.

VAGs	Description/functions	IMT29408	IMT8073
Adhesins			
adhesin v1	AidA-I adhesin involved in diffuse adherence	-	+
aidA	Adhesin involved in diffuse adherence	+	+
csgA	Major subunit of curlin	+	+
eae	Intimin	-	+
eae_theta	Intimin subtype	-	+
fimC	Subunit of Type 1 fimbriae	+	+
fimF	Subunit of Type 1 fimbriae	+	+
fimG	Subunit of Type 1 fimbriae	+	+

fimH	Subunit of Type 1 fimbriae	+	+
matB	Meningitis associated and temperature-regulated fimbriae,B subunit	+	+
paa	Porcine A/E-associated gene	-	+
toxins/hemolysins			
astA(EAST1)	Heat-stable enterotoxin 1	-	+
ehxA	Subunit of enterohemolysin	-	+
ehxB	Subunit of enterohemolysin	-	+
ehxC	Subunit of enterohemolysin	-	+
ehxD	Subunit of enterohemolysin	-	+
hlyA_v1	Haemolysin A	-	+
ibeB/cusC	Copper/silver efflux system	+	+
ibeC/eptC	Phosphoethanolamine transferase	+	+
Serum resistance/ protectins			
iss	Involved in increased serum survival and complement resistance	+	+
ompA	Outer membrane protein A	+	+
ompT	Outer membrane protease VII	+	+
traT	Transfer protein	-	+
miscellaneous genes			
ada	DNA-binding transcriptional dual regulator	+	+
cfa	Cyclopropane fatty acyl phospholipid synthase	+	+
ecpR	Putative transcriptional regulator	+	+
focA	Formate transporter	+	+
malX	Fused maltose and glucose-specific PTS enzyme IIBC components	+	+
map	Type III secretion system (TSS) effector	-	+
slyA	DNA-binding transcriptional activator	+	+
escC	Involved in formation of the type III secretion system (TSS) apparatus	-	+
escD	Involved in formation of the type III secretion system (TSS) apparatus	-	+
escF	Involved in formation of the type III secretion system (TSS) apparatus	-	+
escJ	Involved in formation of the type III secretion system (TSS) apparatus	-	+
escN	Involved in formation of the type III secretion system (TSS) apparatus	-	+
escV	Involved in formation of the type III secretion system (TSS) apparatus	-	+
escR	Involved in formation of the type III secretion system (TSS) apparatus	-	+
escS	Involved in formation of the type III secretion system	-	+

	(TSS) apparatus		
escT	Involved in formation of the type III secretion system (TSS) apparatus	-	+
escU	Involved in formation of the type III secretion system (TSS) apparatus	-	+
espA	A secreted protein	-	+
espD	A secreted protein	-	+
espG	A secreted protein	-	+
espH	A secreted protein	-	+
espK	A secreted protein	-	+
etsA	putative ABC transport system	-	+
nleA	Non-LEE-encoded effector	-	+
nleB1	Non-LEE-encoded effector	-	+
nleB2	Non-LEE-encoded effector	-	+
nleE	Non-LEE-encoded effector	-	+
nleF	Non-LEE-encoded effector	-	+
nleH1	Non-LEE-encoded effector	-	+
nleH2	Non-LEE-encoded effector	-	+
sepD	S-adenosylmethionine decarboxylase	-	+
sepL	S-adenosylmethionine decarboxylase	-	+
sepQ	S-adenosylmethionine decarboxylase	-	+
stcE	Zinc metalloprotease	-	+

"-": negative; "+": positive

3.1.2. Antibiotic resistance genes screening

E. coli IMT29408 harbors two antibacterial resistance genes, *tetA* and *aadA5* (Table 3-2), out of forty-five target sequences screened (Supplemental table 2), which are involved in resistance against tetracycline and streptomycin-spectinomycin correspondingly.

As for IMT8073, there are four out of forty-five antibiotic resistant genes carried on the genome (Table 3-2), bla_{TEM-1}, *sul2, strA* and *strB.* Among them, bla_{TEM-1} is responsible for resistance to ampicillin. *Sul2* codes for resistance against sulfonamide whilst *strA* and *strB* are confined to streptomycin resistance.

Table 3-2. Antibiotic resistance genes present in IMT29408 and IMT8073

Antibiotic resistant genes	Drug name confined by antibiotic resistance gene	IMT29408	IMT8073
aadA5	Streptomycin-spectinomycin	+	-
strA	Streptomycin	-	+
strB	Streptomycin	-	+
sul2	Sulfonamide	-	+
bla_{TEM-1}	Ampicillin	-	+
tetA	Tetracycline	+	-

"-": negative; "+": positive

3.2. Zinc sensitivity test

The antibacterial effects of various concentrations of zinc on the bacterial growth of *E. coli* IMT29408, K-12 and IMT8073 were detected after 24 h incubation (Figure 3-1 and 3-2). Within all three replicates of the experiment, the lowest concentration inhibiting visible bacterial growth (MIC_{zinc}) was 2 mM for IMT29408 and IMT8073, while MIC_{zinc} for K-12 it was determined as 1 mM.

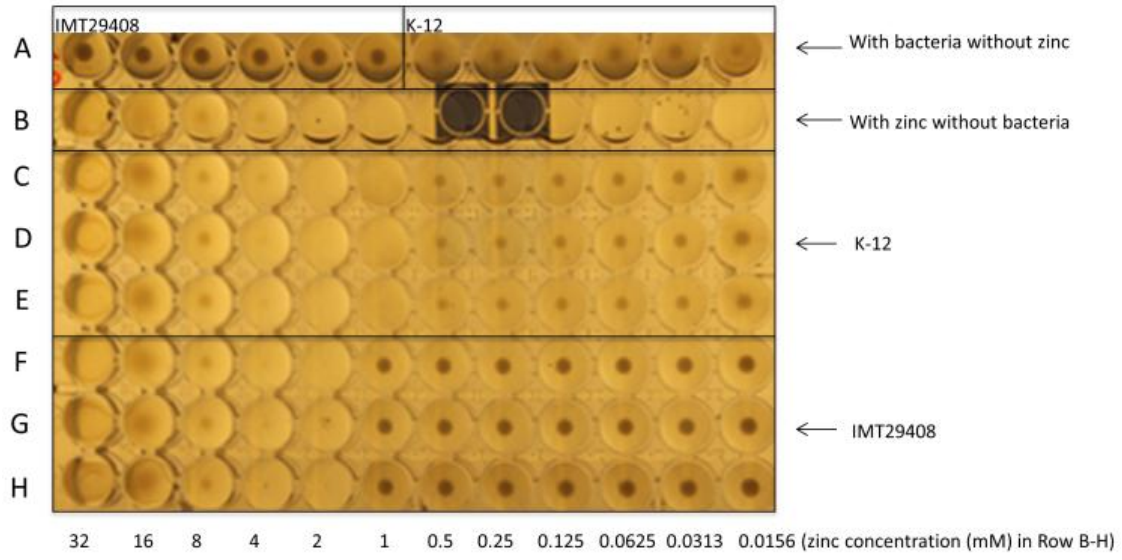


Figure 3-1. Results of zinc sensitivity test (MIC_{zinc}) for IMT29408 and K-12.

Row A: positive control (growth control), first six wells inoculated with IMT29408, last six wells inoculated with K-12; Row B-H coated with various concentrations of zinc chloride. Row B was not inoculated with bacteria as negative control; Row C-E inoculated with K-12 while row F-H inoculated with IMT29408. The bacteria were cultured in LB medium with or without various concentrations of zinc chloride at 37°C for 24 h in anaerobic condition.

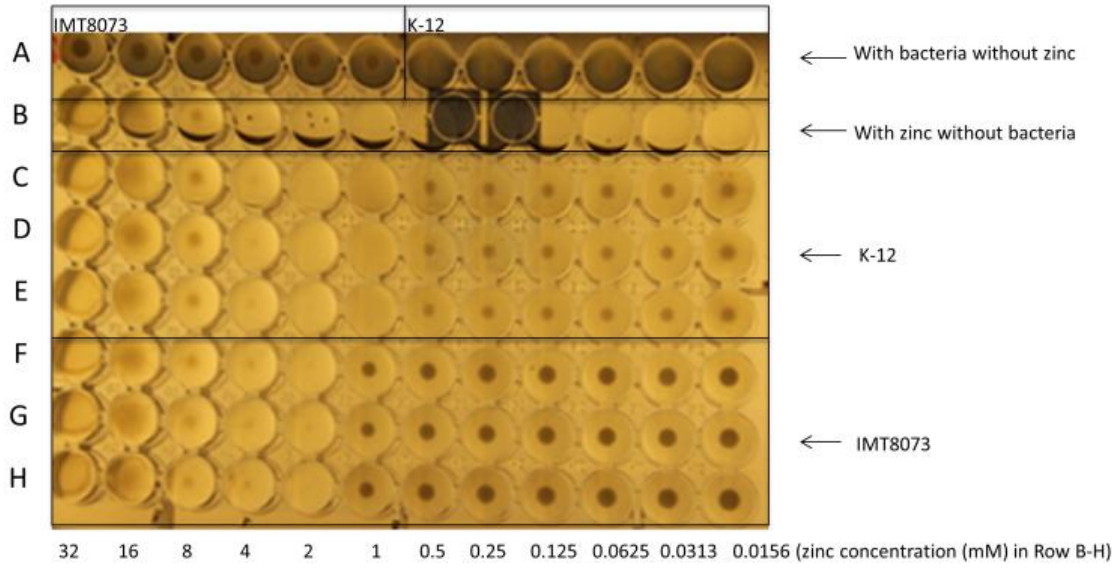


Figure 3-2. Results of zinc sensitivity test (MIC_{zinc}) for IMT8073 and K-12.

Row A: positive control (growth control), first six wells inoculated with IMT8073, last six wells inoculated with K-12; Row B-H coated with various concentrations of zinc chloride. Row B was not inoculated with bacteria as negative control. Row C-E inoculated with K-12 while row F-H inoculated with IMT8073. The bacteria were cultured in LB medium with or without various concentrations of zinc chloride and the plates were incubated at 37°C for 24 h under anaerobic condition.

3.3. Bioreactor culture

3.3.1. Influence of zinc on growth kinetics

Based on the results of the zinc sensitivity test, 1.0 mM zinc was chosen to investigate its effects on growth kinetics in IMT29408 and IMT8073.

In IMT29408, CFU/ml at the starting time of the experiments was determined to range between 3.6 - 4.6 x 10^7 CFU/ml bacteria in the control and zinc exposed samples, respectively. From the CFU/ml curves (Figure 3-3), we observed that the bacterial population in zinc treatment was slightly lower at 1 h compared to LB medium, after that the bacteria progressively multiplied closely and overlapped the control at 3 h and 4 h. After 5 h incubation, the curves began to diverge and exceeded the control, reaching the statistical significance ($p<0.05$) at 7 h of incubation.

Based on the OD_{600} values shown in Figure 3-3, the bacterial turbidities appeared to be lower in zinc treated cases than those of control from 1 h to 6 h while at 7 h it reversed but did not

reach the level of significance between zinc treatment and control.

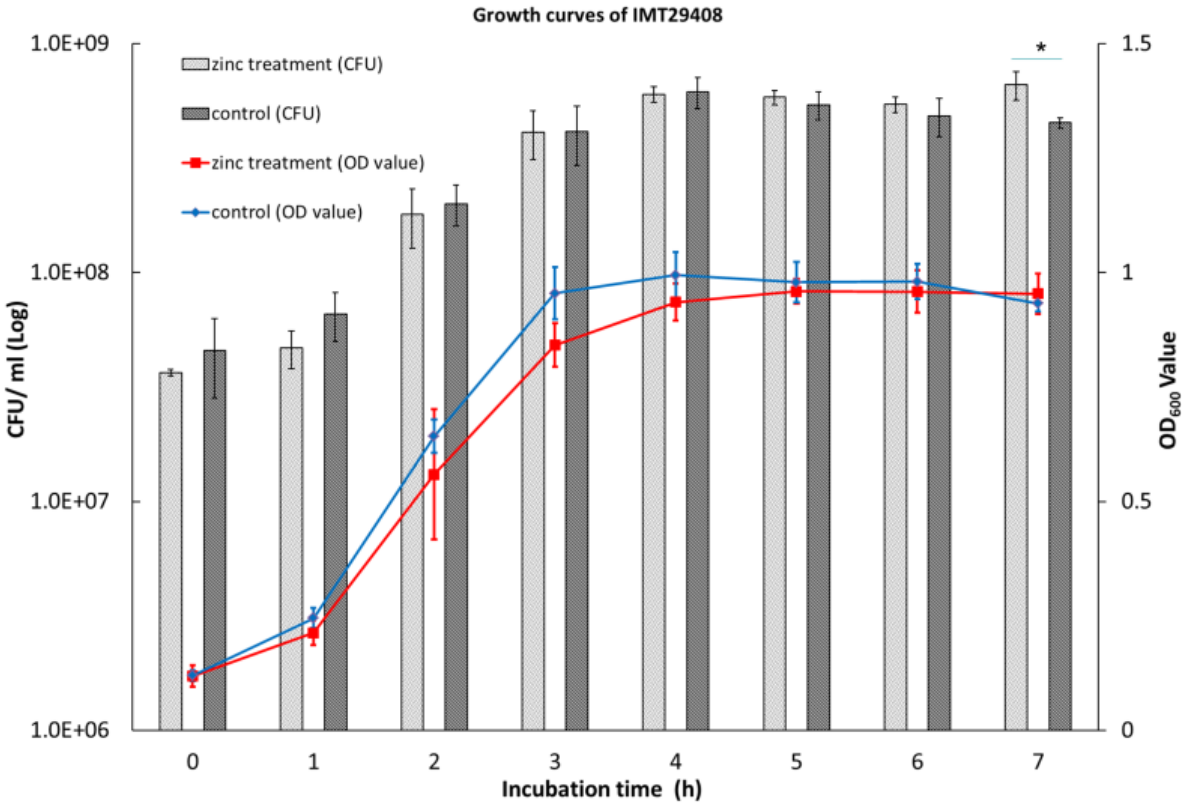


Figure 3-3. Effects of zinc on growth of IMT29408 measured as CFU/ml and OD_{600} values.

Bar: the growth curves of IMT29408 in the presence or absence of 1 mM zinc based on CFUs/ml. Each error bar represents the mean value of three independent replicates ± standard deviation (student's t-test). Line: the growth curves of IMT29408 in the presence or absence of 1 mM zinc on the basis of OD_{600} values. Each error plot represents the mean value of three independent replicates ± standard deviation (student's t-test). "*" representing that CFU/ml between zinc treatment and control displayed significant difference ($p<0.05$). The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition with 200 rpm stirring.

In IMT8073, there was about 3.52 x 10^7 CFU/ml incubated in 1 mM zinc treated medium against 1.2 x 10^7 CFU/ml incubated in control LB medium. From the curves in light of CFU/ml (Figure 3-4), the number of bacterial cells observed in control LB medium was lower than that cultured in the presence of 1 mM zinc at 1 h, while it outnumbered the figure shown in zinc treatment at 2 h and remained until 3 h. From 4 h to 7 h, the bacterial numbers displayed in control were lower than those cultured in zinc treated medium. Although the curves displayed fluctuations, there were no significant differences displayed between zinc

treatment and control from 1 h to 7 h.

However, the OD_{600} values obtained from control LB medium were higher than those from zinc treatment, displaying significant differences between both conditions over the whole incubation period from 1 h to 7 h ($p<0.05$) as indicated in Figure 3-4.

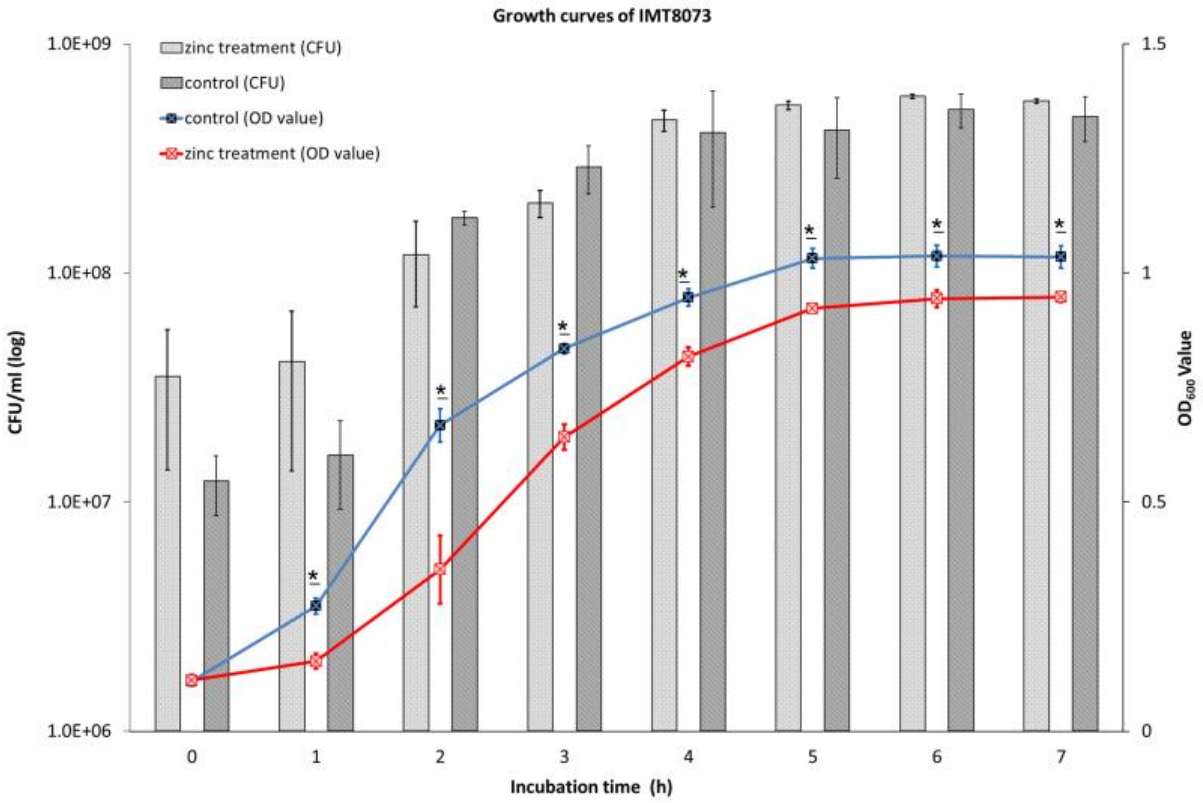


Figure 3-4. Effects of zinc on growth of IMT8073 measured as CFU/ml and OD_{600} value.

Bar: the growth curves of IMT8073 in the presence or absence of 1 mM zinc based on CFU/ml. Each error bar represents the mean value of three independent replicates ± standard deviation (student's t-test). Line: the growth curves of IMT8073 in the presence or absence of 1 mM zinc on the basis of OD_{600} values. Each error plot represents the mean value of three independent replicates ± standard deviation (student's t-test). "*" representing that OD values between zinc treatment and control showed significant difference ($p<0.05$). The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition with 200 rpm stirring.

3.3.2. Influence of zinc on generation time

We calculated the generation time of IMT29408 and IMT8073 in zinc treatment and control conditions during the log phase, the results were shown in Table 3-3.

Table 3-3. The generation time calculated during log phase of IMT29408 and IMT8073

Time period	IMT29408		IMT8073	
	Generation time (min)		Generation time (min)	
	zinc treatment	control	zinc treatment	control
1-2 h	31	38	39	18
2-3 h	51	58	80	82
3-4 h	109	105	50	121

The generation time was calculated as the equation: t/3.3 log (b/B) (t: time interval in minutes; b: means number of bacteria in three independent replicates at the end of the time interval; B: means number of bacteria in three independent replicates at the beginning of time interval). The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition.

3.3.3. Intracellular zinc content measurement

3.3.3.a Calibration curve

The calibration curve was plotted using zinc standard solutions with known zinc concentration of 1.0 g/l. The calibration standard curve used in the present investigation was shown in Figure 3-5. The equation of linear regression, y=0.3295843x-0.0033471 (y=absorbance [$OD_{213.857}$], x=[zinc concentration]) with a linearity (correlation coefficient R^2=0.9973) was applied for this test. The concentration for detection limit by ASS was 0.0117 mg/l, whilst the limit for quantitation concentration was 0.0352 mg/l.

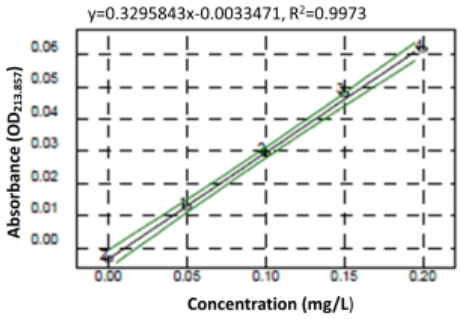


Figure 3-5. Calibration standard curve established by AAS using variable zinc standard solutions.

The concentrations of variable zinc standard solutions were 0.05 mg/l, 0.10 mg/l, 0.15 mg/l, and 0.20 mg/l; X-axis representing the concentrations of calibration standard solutions; Y-axis means corresponding absorbance ($OD_{213.857}$ value).

3.3.3.b Intracellular zinc content in IMT29408

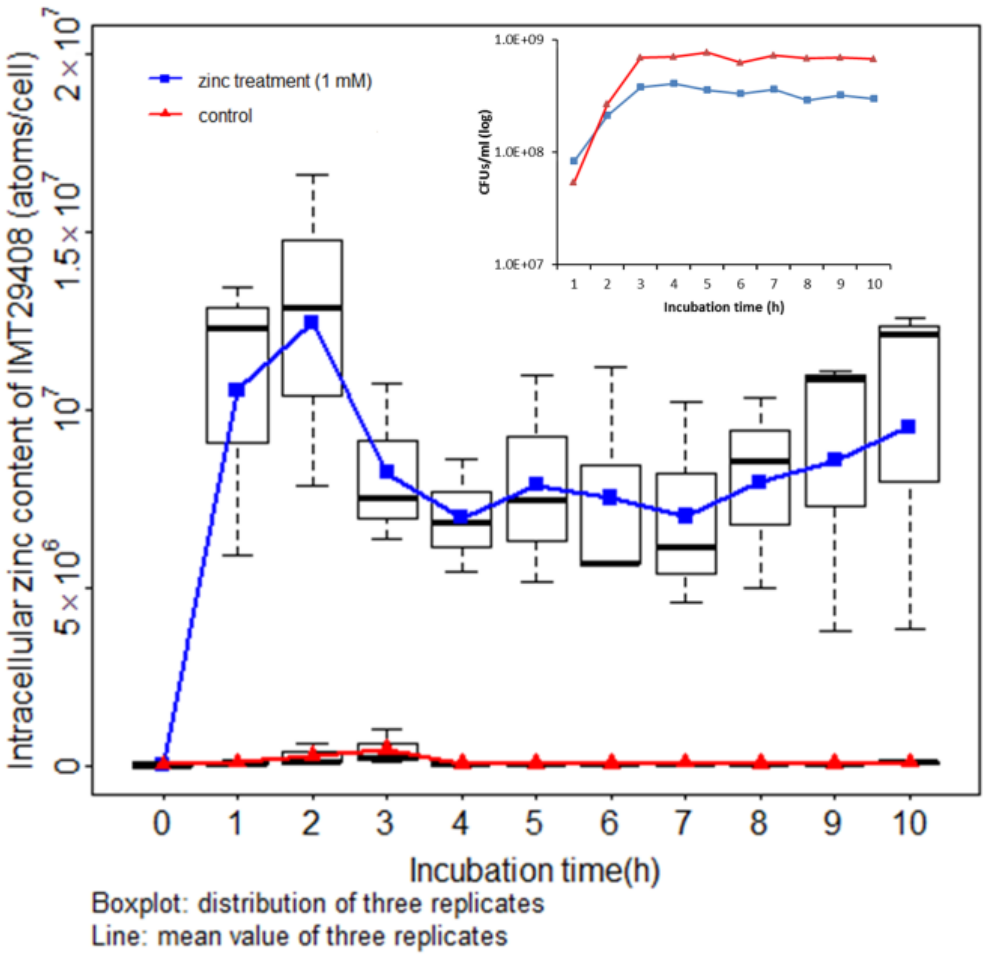


Figure 3-6. Intracellular zinc content of IMT29408 incubated with or without zinc.

In the figure, boxplots representing the distribution of three independent replicates (maximum value, median value and minimum value). The lines represent the mean value of the three independent replicates. The inset shows the CFU/ml of IMT29408 cultured under zinc treatment and control conditions, respectively. The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition with 200 rpm stirring.

At the starting time point (0 h), there was 5 x 10^7 CFU/ml in average incubated in both the 1 mM zinc-containing medium and control medium. The intracellular zinc of the inoculum was calculated to be between 1.3-1.8 x 10^4 atoms/cell. The curves of intracellular zinc content per cell over the measuring period of 0-10 h were shown in Figure 3-6. Under zinc treatment condition, we observed an increase of intracellular zinc content per cell to a maximum of 1.2 x 10^7 atoms/cell at 2 h, a subsequent decrease to 8.2 x 10^6 atoms/cell at 3 h and then maintained between 6.9 x 10^6 to 9.5 x 10^6 atoms/cell during the rest of the observation period.

In contrast, the maximum amount of zinc per cell in control LB medium appeared at 3 h, 4.5 x 10^5 atoms/cell. After that, it declined and fluctuated with a range of 3.7 x 10^4 to 6.1 x 10^4 atoms/cell between 4 h and 10 h.

3.4. 2D-DIGE

According to growth rates in combination with intracellular zinc content results mentioned above, we observed that at 2 h representing exponential phase the OD_{600} value from growth curves in IMT8073 and intracellular zinc content per cell in IMT29408 displayed significant differences between zinc treatment and control, and at 5 h the bacteria in both strains were at the early stationary phase from growth rates. Therefore, we chose 2 h and 5 h time points as the investigated time points for proteomic analysis in both *E. coli* IMT29408 and IMT8073.

3.4.1. Protein concentration confirmation

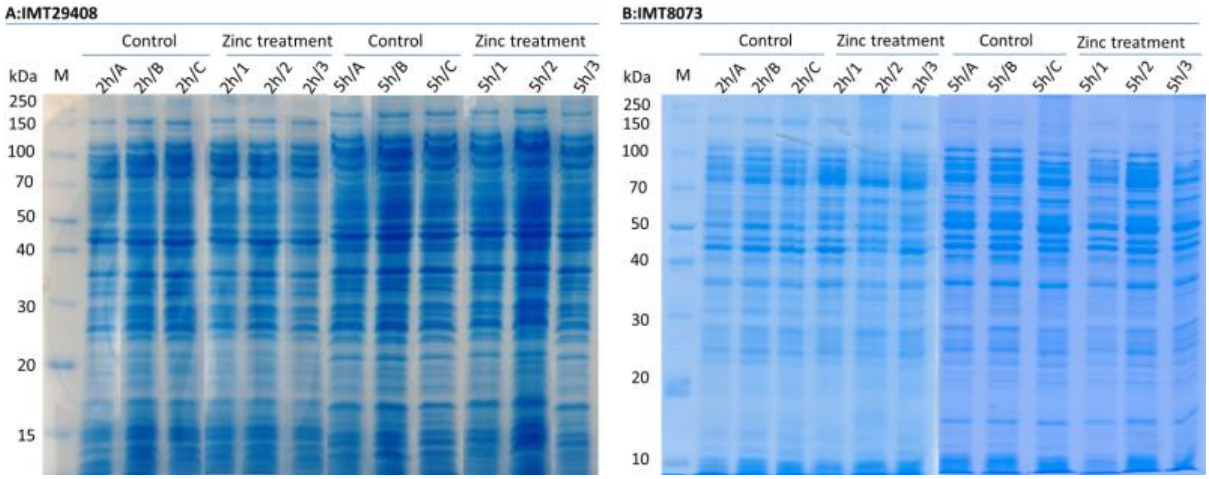


Figure 3-7. Confirmation of extracted protein concentration in IMT29408 (A) and IMT8073 (B).

The letters: A, B and C, represent three independent replicates in control medium; the numbers: 1, 2 and 3, represent three independent replicates in zinc treatment; M: prestained protein marker. This information of samples was corresponding to Table 2-5 for IMT29408 and Table 2-6 for IMT8073. The bacterial protein samples were extracted at 2 h and 5 h from *E. coli* IMT29408 or IMT8073. The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition.

To confirm the consistency of protein concentration measured by the Bradford method, the

volume containing 10 µg proteins from each sample were taken and separated on a SDS-PAGE and visualized with coomassie brilliant blue staining (CBB). The intensity of protein bands among the samples was found to be comparable and consistent (Figure 3-7A and 3-7B).

3.4.2. Results of DIGE

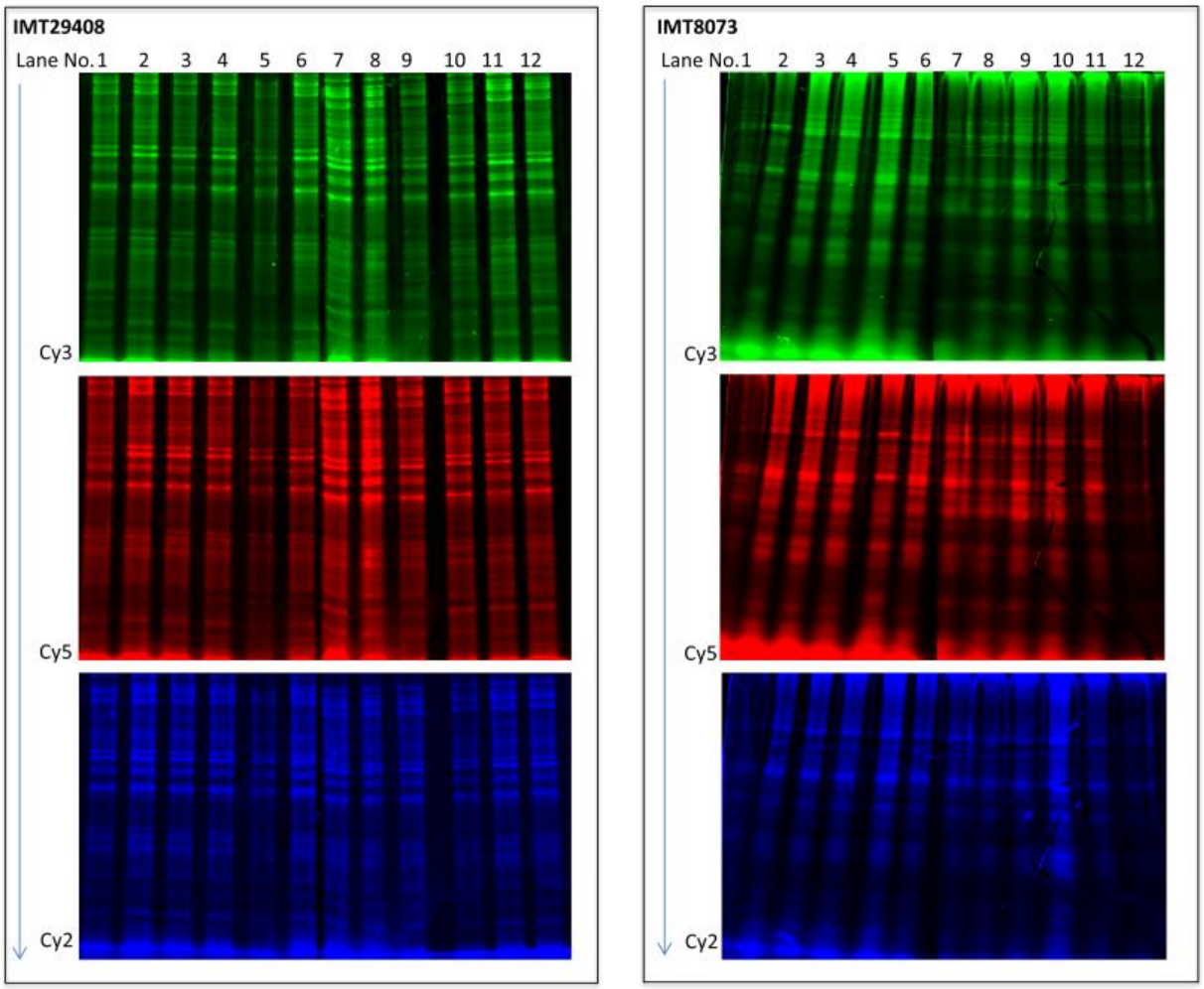


Figure 3-8. Evaluation of dye labeling test in IMT29408 (A) and IMT8073 (B)

The Lanes No.1-12 correspond to respective 2D-DIGE Gel No.1-12 depicted in Table 2-5 for IMT29408 and Table 2-6 for IMT8073 for evaluation of the dye labeling in each gel. Figure 3-8.A: Gel lanes No. 1-6 represent samples collected at 2 h while Gel lanes No. 7-12: samples collected at 5 h in IMT29408; Figure 3-8.B: Gel lanes No. 1-6: samples collected at 2 h while Gel lanes No. 7-12: samples collected at 5 h in IMT8073. The bacterial protein samples were extracted at 2 h and 5 h from *E. coli* IMT29408 or IMT8073. The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition with 200 rpm stirring.

The evaluation of the efficiency and consistency of dye-labeling from each gel was visualized on SDS-PAGE gels seen in Figure 3-8 above.

2D-DIGE images of the complete experiments were shown in the supplementary material (Supplemental figure 1). A representative gel image was shown in the Figure 3-9.

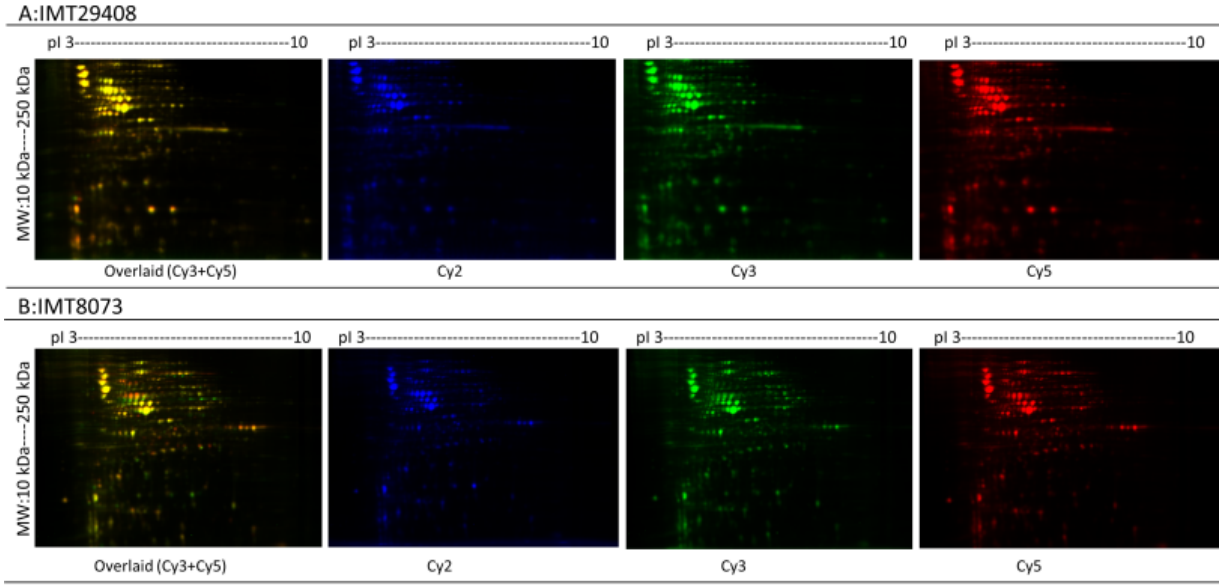


Figure 3-9. Representative 2D-DIGE images of three dyes (Cy2, Cy3 and Cy5) from IMT29408 (A) and IMT8073 (B).

Figure 3-9.A: Images from IMT29408 at 5 h (source: Gel 7); Figure 3-9.B: Images from IMT8073 at 2 h (source: Gel 1). Taken overlaid image in Figure 3-9.B as a representative, zinc treatment sample labeled with Cy5 (red), control sample labeled with Cy3 (green); Red spots indicate more expression of proteins induced by zinc, green spots represent more expression of proteins cultured in LB medium (control), and yellow spots (overlapped color) show proteins that are similarly expressed in both zinc treatment and control. The bacterial protein samples were extracted at 2 h and 5 h from *E. coli* IMT29408 or IMT8073. The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition with 200 rpm stirring.

3.5. Quantitative gel analysis

In this study, we compared protein expression at two time points of incubation (2 h zinc vs. 2 h control, and 5 h zinc vs. 5 h control) to investigate the effects of zinc on protein expression of *E. coli* compared to absence of zinc treatment. In addition, the protein expressional differences between the culture time points (2 h vs. 5 h) of the exposure was also

analyzed and grouped as “2 h zinc vs 5 h zinc only”, “shared between 2 h zinc vs 5 h zinc and 2 h control vs. 5 h control”, and “2 h control vs. 5 h control only” in order to explore the effects of persistent zinc treatment (from 2 h to 5 h) on protein expression.

Delta2D software analysis of IMT29408 revealed a total of 544 protein spots with unique ID across all the images (Figure 3-10). Out of these spots, there were 172 protein spots differentially expressed (student‘s *t*-test, $p<0.05$) among the five compared groups (“2 h zinc vs. 2 h control” group; “5 h zinc vs. 5 h control” group; “2 h zinc vs 5 h zinc only” group, “shared between 2 h zinc vs 5 h zinc and 2 h control vs. 5 h control” group, and “2 h control vs. 5 h control only” group), the distribution of all these differentially expressed spots was summarized in Table 3-4. Out of those, 2 h of zinc exposure resulted in differentially expression of 17 spots (4 up-regulated and 13 down-regulated), while 5 h of zinc exposure resulted in 19 differentially expressed spots (12 up-regulated and 7 down-regulated). There were four spots up-regulated simultaneously at both 2 h and 5 h of zinc treatment according to the spot ID.

In addition, 70 spots (31 up-regulated and 39 down-regulated) were differentially expressed only during the course of time from 2 to 5 h of zinc exposure. 62 spots (36 up-regulated and 26 down-regulated) differentially expressed were found to be common between the zinc and control exposure group. 28 spots (8 up-regulated and 20 down-regulated) appeared to be differentially expressed only in controls from 2 h to 5 h.

Delta2D software analysis of IMT8073 indicated a total of 413 spots across all the 2D-DIGE images (Figure 3-11). Out of them, 200 spots were investigated as differentially expressed among the five compared groups (“2 h zinc vs. 2 h control” group; “5 h zinc vs. 5 h control” group; “2 h zinc vs 5 h zinc only” group, “shared between 2 h zinc vs 5 h zinc and 2 h control vs. 5 h control” group, and “2 h control vs. 5 h control only” group). The distribution of these spots was shown in Table 3-4. Specifically, 2 spots were up-regulated as an influence of zinc at 2 h. After 5 h of incubation with zinc, there were 18 spots up-regulated and 8 spots down-regulated.

Zinc exposure of 2 h to 5 h appeared to influence 97 spots (20 up-regulated and 77 down-regulated). In addition, 54 spots (25 up-regulated and 29 down-regulated) were found to be commonly distributed between the zinc and control exposure group. 36 spots (8 up-regulated and 28 down-regulated) appeared to be differentially expressed only in controls from 2 h to 5 h.

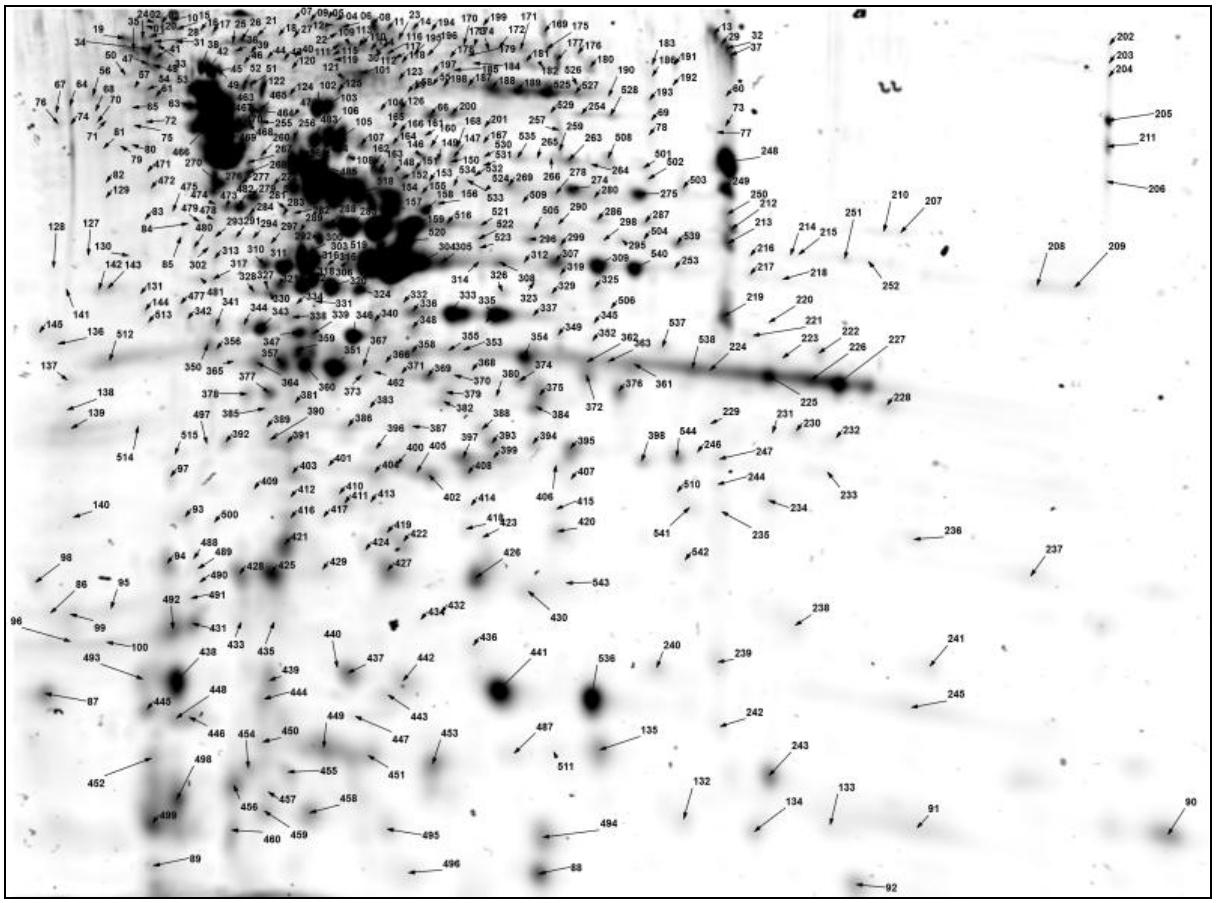


Figure 3-10. Overview of all the detected protein spots indicated as unique ID number across all the 2D-DIGE images in IMT29408.

The 2-DE image is a fused artificial image using the Delta2D software containing all the protein spots (with unique ID number) across all the 2D-DIGE images. The bacterial protein samples were extracted at 2 h and 5 h from *E. coli* IMT29408. The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition with 200 rpm stirring.

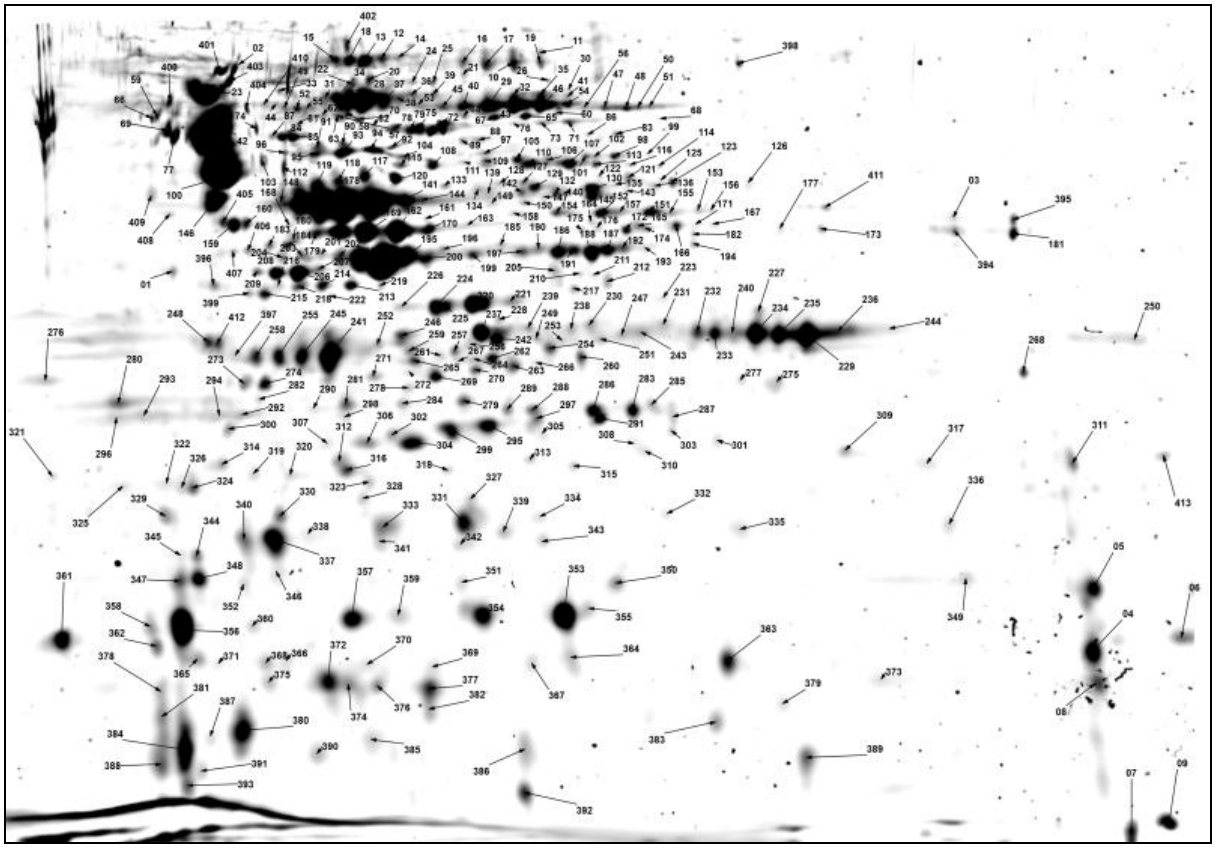


Figure 3-11. Overview of all the detected protein spots indicated as unique ID number across all the 2D-DIGE images in IMT8073.

The 2-DE image is a fused artificial image using the Delta2D software containing all the protein spots (with unique ID number) across all the 2D-DIGE images. The bacterial protein samples were extracted at 2 h and 5 h from *E. coli* IMT8073. The bacteria were cultured using bioreactor in LB medium (pH 6.0) with or without 1 mM zinc chloride at 37°C under anaerobic condition with 200 rpm stirring.

Table 3-4. The number of differentially expressed protein spots of IMT29408 and IMT8073 distributed in each compared group.

Item	**No.of differentially expressed protein spots**	
	Up-regulated	**Down-regulated**
***E. coli* IMT29408**		
2 h zinc vs. 2 h control	4	13
5 h zinc vs. 5 h control	12	7
2 h zinc vs. 5 h zinc only	31	39
Shared spots between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control	36	26

2 h control vs. 5 h control only	8	20
***E. coli* IMT8073**		
2 h zinc vs. 2 h control	2	0
5 h zinc vs. 5h control	18	8
2 h zinc vs. 5 h zinc only	20	77
Shared spots between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control	25	29
2 h control vs. 5 h control only	8	28

As for some differentially expressed spots which distributed in different compared groups according to the spot ID, these spots were counted once when we calculated the total number of all the differentially expressed spots among the five compared groups.

3.6. Protein identification

3.6.1. Excision of differentially expressed protein spots

In IMT29408, among 172 differentially expressed spots distubuted in the five compared groups above mentioned, there were 146 protein spots chosen for further identification after exclusion of these differentially expressed spots distributed in “2 h control vs. 5 h control only” group, as these protein spots were not influenced by zinc. As a consequence, 117 differentially expressed spots out of 146 were excised from 2-DE gels.

As for IMT8073, among the 200 differentially expressed spots, 168 spots were considered as target spots for further analysis (excluded the spots distributed in “2 h control vs. 5 h control only” group). Out of them, 157 spots were excised from 2-DE gels.

3.6.2. Proteins identified

IMT29408

There were 73 protein spots identified out of 117 excised spots (Supplemental table 3), and a total of 54 proteins were identified due to some proteins represented by more than one analyzed spot (Table 3-5).

As for identified proteins in “shared between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control” group, although these proteins were differentially expressed over time and were with

different fold changes between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control, they were indicated as normal changes during growth irrespective of zinc treatment. Thus, we did not emphasize on these proteins for further analysis.

As a consequence, a total of 35 identified proteins, including 2 up-regulated and 7 down-regulated in "2 h zinc vs. 2 h control" group, 7 up-regulated and 3 down-regulated in "5 h zinc vs. 5 h control" group, and 6 up-regulated and 14 down-regulated in "2 h zinc vs. 5 h zinc only" group, were regarded as aimed proteins for identification and further analysis (Table 3-6) and marked on 2-DE gel in Figure 3-12.

Table 3-5. Distribution of detailed number of differentially expressed protein spots identified and number of identified proteins in IMT29408 and IMT8073, respectively.

Item	**Up-regulated**	**Down-regulated**
	No. of identified spots / No. of identified proteins	**No. of identified spots / No. of identified proteins**
***E. coli* IMT2908**		
2 h zinc vs. 2 h control	2 / 2	8 / 7
5 h zinc vs. 5 h control	8 / 7	3 / 3
2 h zinc vs. 5 h zinc only	14 / 6	21 / 14
Shared between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control	19 / 16	11 / 10
***E. coli* IMT8073**		
2 h zinc vs. 2 h control	2 / 2	0
5 h zinc vs. 5 h control	14 / 11	6 / 6
2 h zinc vs. 5 h zinc only	13 / 8	56 / 26
Shared between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control	17 / 10	13 / 11

As for some differentially expressed protein spots distributed in different compared groups according to the spot ID, we excised and identified these protein spots only once. Due to the post translation modifications, some of the identified proteins appeared simultaneously in both "2 h zinc vs. 5 h zinc only" group and "shared between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control" group, these proteins were categorized as proteins of the shared group.

Table 3-6. Differentially expressed proteins identified in IMT29408.

Gene name	Protein name	Descripton of functions	Fold change
2 h zinc vs. 2 h control			
degP(htrA) [#]	Serine endoprotease	Stress defense, degradation of damaged proteins	3.1
fabF [#]	3-oxoacyl-(acyl carrier protein) synthase II	Fatty acid biosynthetic process	2
manX	PTS system mannose-specific transporter subunit IIAB	Transmembrane transport of mannose to cytoplasm	-1.6
mdh [&]	Malate dehydrogenase	Tricarboxylic acid cycle, anaerobic respiration	-2
pgk	Phosphoglycerate kinase	Glycolysis	-2.8
	Phosphoglycerate kinase	Glycolysis	-2.2
rbsB	D-ribose transporter subunit RbsB	Component of the ribose ABC transporter	-1.6
rpoA	DNA-directed RNA polymerase, alpha subunit	Transcription, zinc ion binding	-3.1
tsf	Translation elongation factor Ts	Translational elongation, zinc ion binding	-1.5
yhdH	Dehydrogenase	Oxidation-reduction process, zinc ion binding	-1.6
5 h zinc vs. 5 h control			
degP(htrA) [#]	Serine endoprotease	Stress defense, degradation of damaged proteins	3.8
fabF [#]	3-oxoacyl-(acyl carrier protein) synthase II	Fatty acid biosynthetic process	2.1
rplD	50S ribosomal protein L4	Translation, invovled in stress response	2.4
tnaA	Tryptophanase	Cellular amino acid metabolic, catabolic process	1.8
tpiA	Triosephosphate isomerase	Glycolysis	1.9
	Ttriosephosphate isomerase	Glycolysis	2.1
tsx	Nucleoside-specific channel-forming protein	Transpot to amino acid,deoxynucleosides	1.7
ychF	GTP-binding protein YchF	ATP catabolic process, negatively stress defense	2.1
ahpC	Peroxiredoxin	Oxidative stress defense	-2.3
pflB	Formate acetyltransferase	Glucose metabolic process, anarobic respiration	-1.7
rffG	dTDP-glucose 4,6-dehydratase	Nucleotide sugar biosynthesis	-1.5
2 h zinc vs 5 h zinc only			
argS	Arginyl-tRNA synthetase	Protein translation, nucleoside binding	2
asnS	Asparagine--tRNA ligase	Translation, necleotide binding	1.7

fabI	Enoyl-(acyl carrier protein,ACP) reductase	Fatty acid biosynthesis	1.8
groES	Co-chaperonin GroES	Chaperone, response to oxidative stress	3.3
mdh [&]	Malate dehydrogenase	Tricarboxylic acid cycle, anaerobic respiration	1.6
talB	Transaldolase B	Carbohydrate metabolic process	1.7
accA	Acetyl-CoA carboxylase carboxyltransferase	Fatty acid biosynthesis process	-4.1
adhE	Acetaldehyde dehydrogenase	Oxidaion-reduction process	-1.5
fabF	3-oxoacyl-(acyl carrier protein) synthase II	Fatty acid biosynthetic process	-1.6
fumB	Fumarate hydratase class I, anaerobic	Tricarboxylic acid cycle, anaerobic respiration	-1.8
fusA	Elongation factor G	Protein elongation	-2.7
	Elongation factor G	Protein elongation	-2.2
	Elongation factor G	Protein elongation	-2.1
guaB	Inosine-5'-monophosphate dehydrogenase	Nucleoside biosynthesis	-1.8
hyaB	Hydrogenase 1, large subunit	Oxidaion-reduction process	-3.2
ompC	Outer membrane protein OmpC	Composed of permeability of cells, channels for antibiotic transporting	-2.7
ompX	Outer membrane protein X	Composed of permeability of cells, channels for antibiotic transporting	-3.5
pykA	Pyruvate kinase	Glycolysis (a key enzyme)	-3
rplJ	50S ribosomal protein L10, partial	Post translation	-1.7
serS	Serine--tRNA ligase	tRNA aminoacylation for protein translation	-2.3
surA	Peptidyl-prolyl cis-trans isomerase	Assembly of outer membrane porins	-1.7
tuf	Translation elongation factor Tu, partial	Protein elongation	-2
	Translation elongation factor Tu, partial	Protein elongation	-1.5
	Translation elongation factor Tu	Protein elongation	-1.8

Note: "-" down-regulated. Out of 35 identified proteins, several proteins were identified in more than one analyzed spot with different fold changes, e.g. Pgk and TpiA. In addition, DegP, FabF, and Mdh appeared in different compared groups based on protein spot ID, marked as follows: "[#]": proteins present in both "2 h zinc vs. 2 h control" group and "5 h zinc vs. 5 h control" group; "[&]" proteins present in both "2 h zinc vs. 2 h control" group and "2 h zinc vs. 5 h zinc only" group.

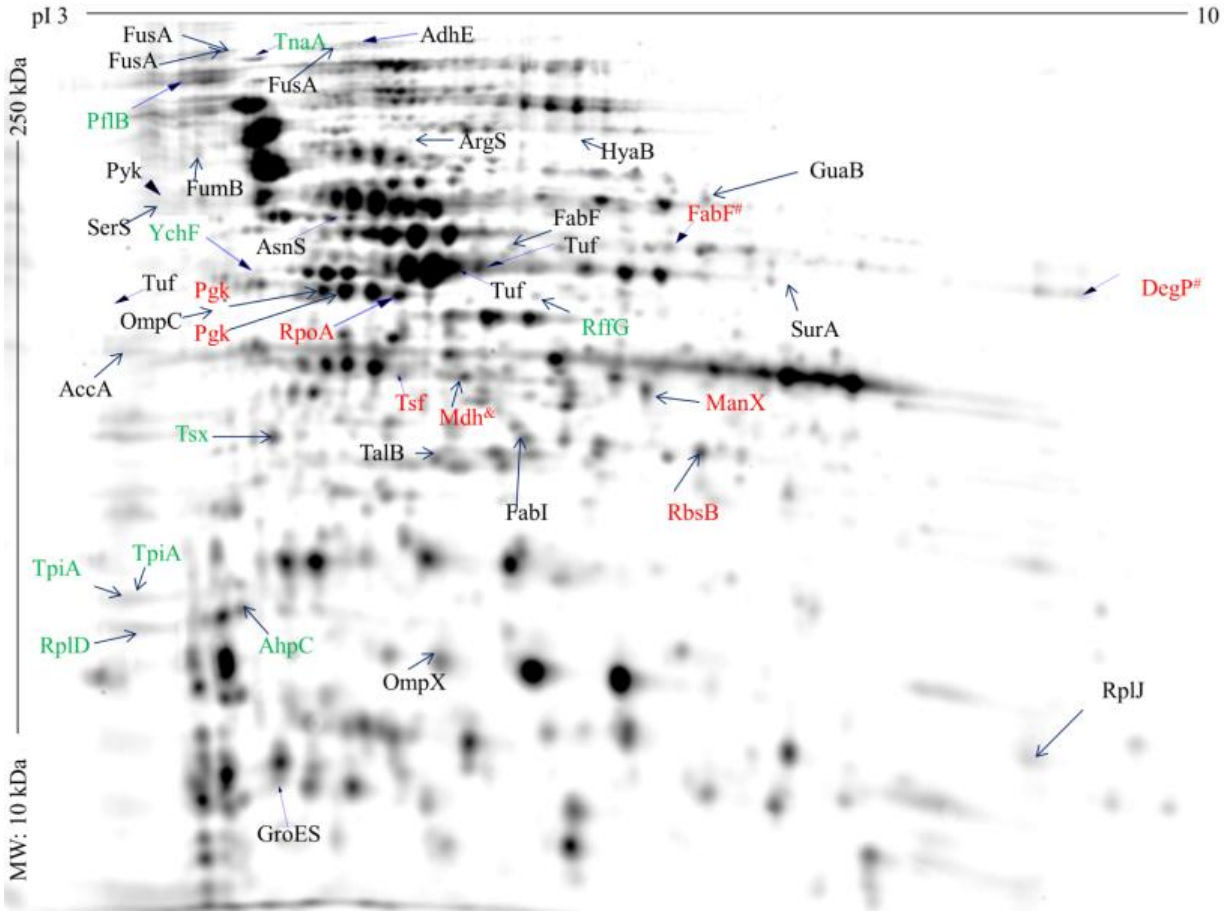


Figure 3-12. Identified proteins in IMT29408 as positioned on 2-DE gel.

Red: proteins identified in "2 h zinc vs. 2 h control" group; Green: proteins identified in "5 h zinc vs. 5 h control" group; Black: proteins identified in "2 h zinc vs. 5 h zinc only" group; "#": proteins present in both "2 h zinc vs. 2 h control" group and "5 h zinc vs. 5 h control" group; "&" proteins present in both "2 h zinc vs. 2 h control" group and "2 h zinc vs. 5 h zinc only" group. The 2-DE image used here originated from Gel No.12 described in Table 2-5.

IMT8073

In IMT8073, 114 of 157 differentially expressed protein spots excised were identified as shown in Table.3-5. Out of 114 identified spots, 65 proteins were identified in "2 h zinc vs. 2 h control" group, "5 h zinc vs. 5 h control" group, "2 h zinc vs. 5 h zinc only" group and "shared between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control "group (Supplemental data 3).

In the end, there were 47 proteins selected for further analysis shown in Table 3-7 and marked on 2-DE gel (Figure 3-13) after ruling out proteins identified in "shared between 2 h zinc vs. 5

h zinc and 2 h control vs. 5 h control" group. Among these 47 identified proteins, there were 2 up-regulated proteins in "2 h zinc vs 2 h control" group, 11 up-regulated and 6 down-regulated proteins in "5 h zinc vs 5 h control" group, and 8 up-regulated and 26 down-regulated proteins in " 2 h zinc vs 5 h zinc only" group.

Table 3-7. Differentially expressed proteins identified in IMT8073.

Gene name	Protein name	Description of functions	Fold change
2 h zinc vs. 2 h control			
clpB	ATP-dependent chaperone protein	Chaperone, response to oxidative stress	2
katE	catalase HPII	Oxidative stress defense	2.6
5 h zinc vs. 5 h control			
accC	Biotin carboxylase	Fatty acid biosythetic process	1.9
asnS	Asparagine--tRNA ligase	tRNA aminoacylation for protein translation	3.6
aspA *	Aspartate ammonia-lyase	Asparate metabolic process	1.8
atpA	ATP synthase alpha subunit	ATP synthase activity,hydrolase activity	1.7
atpD	ATP synthase beta subunit	ATP synthase activity,hydrolase activity	1.6
	ATP synthase beta subunit	ATP synthase activity,hydrolase activity	1.6
	ATP synthase beta subunit	ATP synthase activity,hydrolase activity	1.8
degP(hrtA)	Serine endoprotease	Stress defense, degradation of damaged proteins	2
fabF *	3-oxoacyl-ACP synthase	Fatty acid biosynthetic process	3.9
hybC *	Hydrogenase 2 large subunit	Anarobic respiration	2.3
pyrG	CTP synthase	Pyrimidine biosythesis, glutamine metabolic process	1.8
tnaA	Tryptophanase	Cellular amino acid metabolic, catabolic process	4
	Tryptophanase	Cellular amino acid metabolic, catabolic process	3.9
tsf	Elongation factor Ts	Protein elongation,zinc ion binding	1.5
cadA *	Lysine decarboxylase	Lysine decarboxylase activity	-6.2
minD	Cell division inhibitor MinD, partial	ATPase acivity during cell division	-3.5
nanA	N-acetylneuraminate lyase	N-acetylneuraminate lyase activity	-1.5
nusA *	Transcription termination factor	Transcription regulation	-1.9

pflB *	Formate acetyltransferase	Glucose metabolic process, anarobic respiration	-5.5
udp	Uridine phosphorylase	Uridine phosphorylase activity	-1.6
2 h zinc vs. 5 h zinc only			
aspA *	Aspartate ammonia-lyase	Asparate metabolic process	2.9
	Aspartate ammonia-lyase, partial	Asparate metabolic process	1.9
	Aspartate ammonia-lyase	Asparate metabolic process	2.5
	Aspartate ammonia-lyase domain protein	Asparate metabolic process	3
glnA	Glutamine synthetase	Glutamine metabolic process	3.4
glpQ	Glycerophosphodiester phosphodiesterase	Glycerol metabolic process	5.1
	Glycerophosphodiester phosphodiesterase	Glycerol metabolic process	1.8
groS	Co-chaperonin GroES	Chaperone, response to oxidative	2.6
hybC *	Hydrogenase 2 large subunit	Anarobic respiration	2.1
lysS	Lysyl-tRNA synthetase	tRNA aminoacylation for protein translation	2.4
pdxJ	Pyridoxine 5'-phosphate synthase	Pyridoxine biosynthetic process	1.8
prfB	Peptide chain release factor 2	Translation release factor activity	1.8
adhE	Acetaldehyde dehydrogenase	Oxidaion-reduction process	-1.8
cadA *	Lysine decarboxylase	Lysine decarboxylase activity	-4.1
eutD	Phosphate acetyltransferase	Phosphate acetyltransferase activity	-1.8
fabF *	3-oxoacyl-ACP synthase	Fatty acid biosynthetic process	-1.5
fbaA	Fructose-bisphosphate aldolase	Glycolytic process	-1.8
	Fructose-bisphosphate aldolase	Glycolytic process	-1.9
fumA	Fumarate hydratase, partial	Tricarboxylic acid cycle	-2.5
	Fumarate hydratase	Tricarboxylic acid cycle	-1.8
glmS	Glucosamine--fructose-6-phosphate aminotransferase	Hexosamine biosynthesis.	-2.9
glnS	Glutaminyl-tRNA synthetase	Glutaminyl-tRNA aminoacylation	-3.1
glyS	Glycyl-tRNA synthetase beta subunit, partial	Glycyl-tRNA aminoacylation	-2.3
gnd	6-phosphogluconate dehydrogenase	D-gluconate metabolic process	-2.6
hemX	Uroporphyrinogen III C-methyltransferase	Biosynthetic process	-1.8
minD	Cell division inhibitor MinD, partial	ATPase acivity during cell division	-2.5
nusA *	Transcription termination factor	Transcription regulation	-1.9
oppA	Oligopeptide ABC transporter	Protein transporting	-2.9
pflB *	Formate acetyltransferase	Glucose metabolic process, anarobic respiration	-2.3

	Formate acetyltransferase	Glucose metabolic process, anarobic respiration	-7.3
pnp	Polyribonucleotide nucleotidyltransferase	mRNA degradation	-1.6
	Polyribonucleotide nucleotidyltransferase	mRNA degradation	-1.8
pspE	Thiosulfate sulfurtransferase	Thiosulfate sulfurtransferase activity	-2
pta	Phosphate acetyltransferase	Acetate metobolic process	-2.2
	Phosphate acetyltransferase	Acetate metobolic process	-1.7
pyk	Pyruvate kinase	Glycolysis (a key enzyme)	-3
	Pyruvate kinase	Glycolysis (a key enzyme)	-2.8
rho	Transcription termination factor	Transcription termination	-1.9
rplI	50S ribosomal protein L9	Translation	-3.1
rpoA	DNA-directed RNA polymerase alpha subunit	Transcription, zinc ion binding	-2.9
tdcE	Keto-acid formate acetyltransferase	Arbohydrate metabolic process	-2.5
	Keto-acid formate acetyltransferase	Arbohydrate metabolic process	-2.6
	Keto-acid formate acetyltransferase	Arbohydrate metabolic process	-4
tuf	Translation elongation factor Tu	Protein elongation	-1.7
	Translation elongation factor Tu, partial	Protein elongation	-1.7
	Translation elongation factor Tu, partial	Protein elongation	-1.9
	Translation elongation factor Tu, partial	Protein elongation	-2.1
	Translation elongation factor Tu	Protein elongation	-2.8
typA	GTP-binding protein TypA, partial	Translation	-1.9
ychF	GTP-binding protein	ATPase activity	-1.9

Note: "-" down-regulated. Out of 47 identified proteins, several proteins were identified in more than one analyzed spot with different fold changes, e.g. AtpD and AspA. In addition, six proteins, AspA, FabF, HybC, CadA, NusA and PflB, appeared simultaneously in "5 h zinc vs 5 h control" group and "2 h zinc vs 5 h zinc only" group based on protein spot ID, marked as "*".

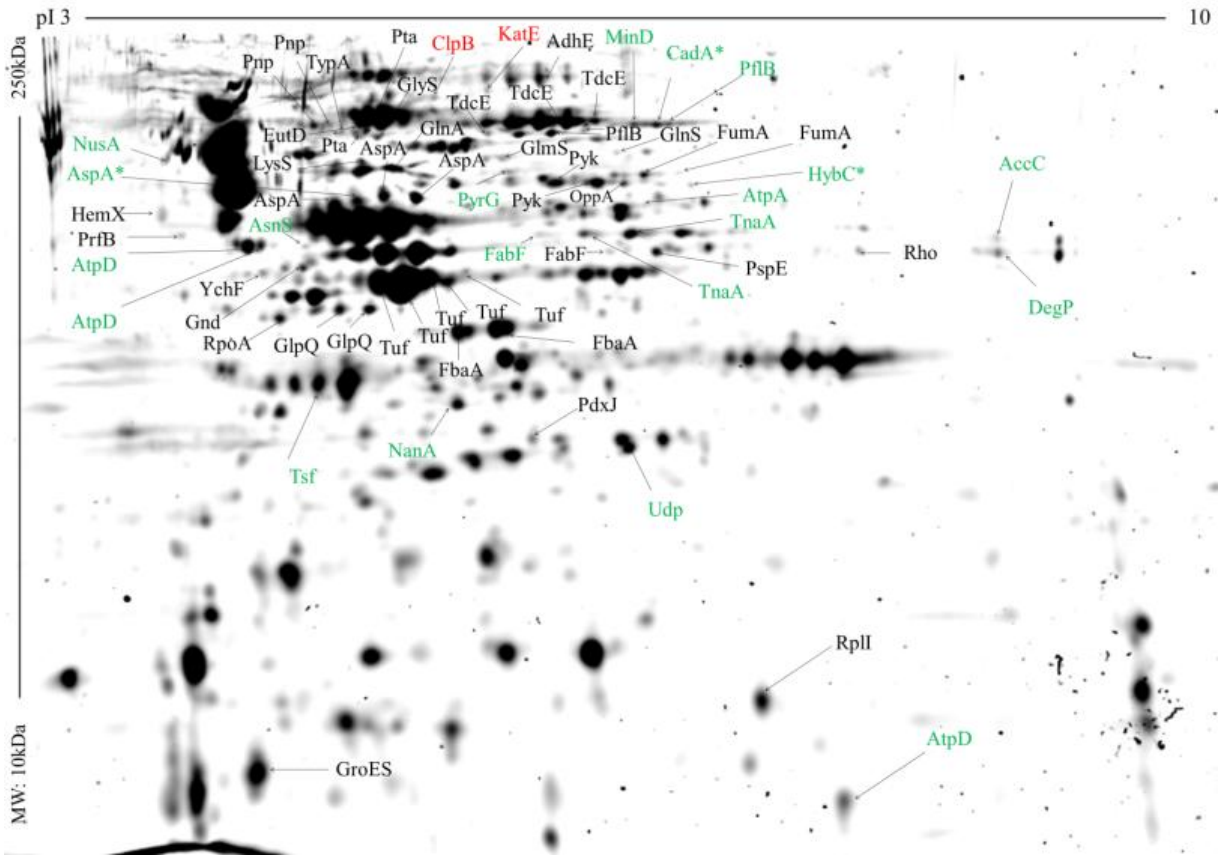


Figure 3-13. Identified proteins in IMT8073 as positioned on 2-DE gel.

Red: proteins identified in "2 h zinc vs. 2 h control" group; Green: proteins identified in "5 h zinc vs. 5 h control" group; Black: proteins identified in "2 h zinc vs. 5 h zinc only" group; "*": proteins present in both "5 h zinc vs. 5 h control" group and "2 h zinc vs. 5 h zinc only" group. The 2-DE image used here originated from the fusion image.

3.7. Comparative proteomics between commensal and pathogenic *E. coli*

Among the differentially expressed and identified proteins, twelve proteins appeared to be comparatively regulated (e.g. DegP, FabF, TnaA and Tuf) in both commensal and pathogenic *E. coli* strains (Table 3-8).

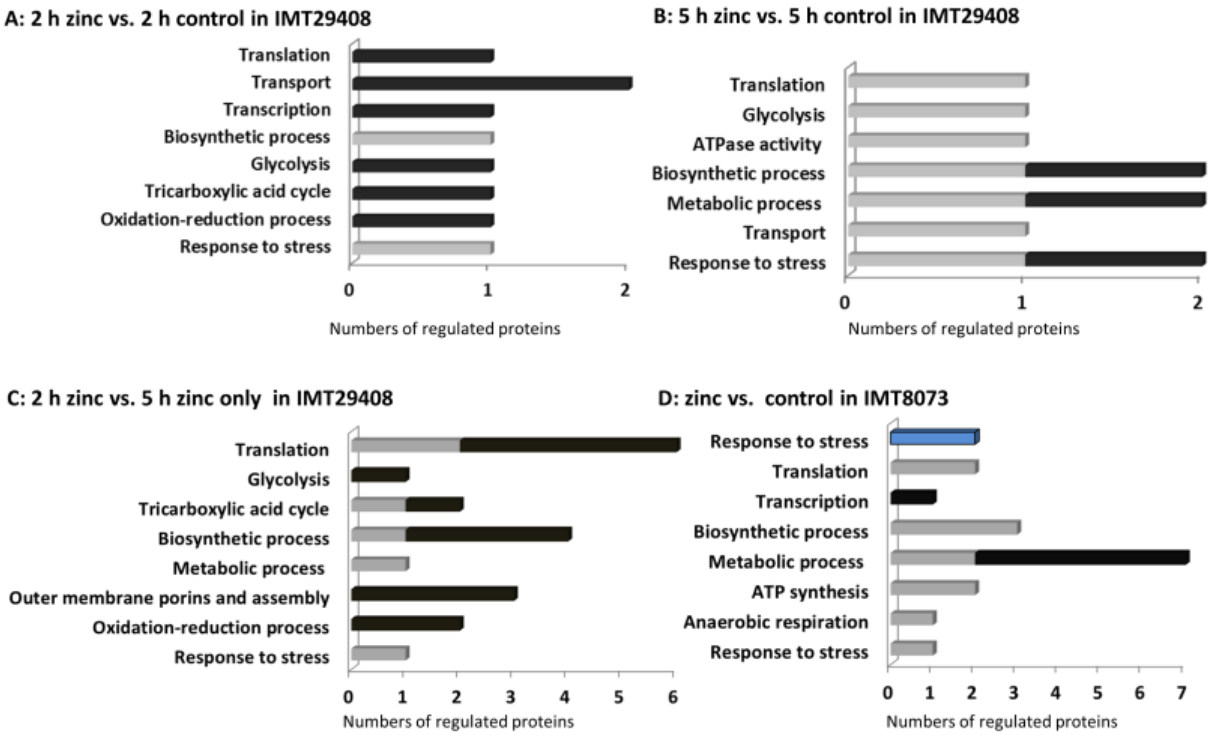
Table 3-8. The same proteins appeared in both IMT29408 and IMT8073.

Gene name	IMT29408			IMT8073		
	2 h	5 h	2 h zinc vs 5 h zinc only	2 h	5 h	2 h zinc vs. 5 h zinc only
degP						
fabF						
tnaA						
ychF						
groES						
asnS						
tsf						
pflB						
rpoA						
tuf						
adhE						
pyk						

Note: Grey: proteins up-regulated; Black: proteins down-regulated. "2 h" presenting "2 h zinc vs. 2 h control" group; "5 h" presenting "5 h zinc vs. 5 h control" group.

3.8. Gene ontology classification

The proteins identified were classified into several groups based on the gene ontology analysis as shown in Figure 3-14.



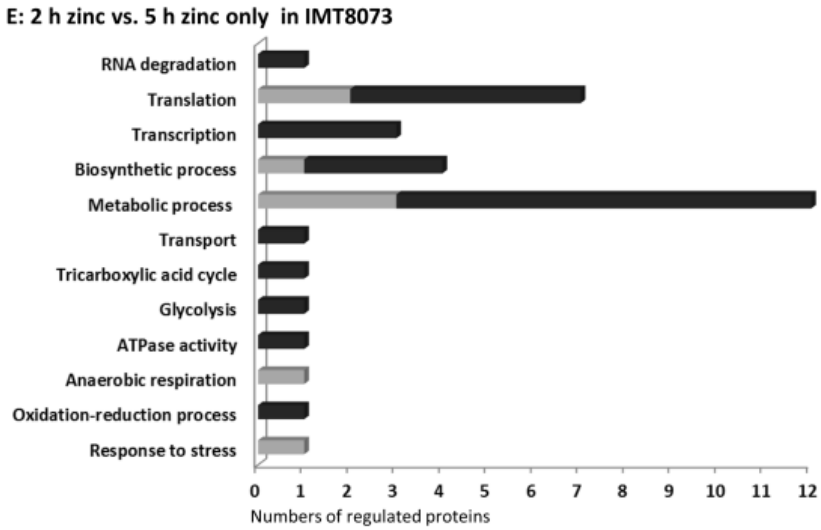


Figure 3-14. Classification of identified proteins according to gene ontology in IMT29408 and IMT8073

Gray bar: up-regulated proteins; Black bar: down-regulated proteins. As for Figure 3-14.D: "zinc vs. control" representing two compared groups: "2 h zinc vs. 2 h control" group and "5 h zinc vs. 5 h control" group; dark gray with black border bar: up-regulated proteins at "2 h zinc vs 2h control" group; light gray bar: up-regulated proteins at "5 h zinc vs. 5 h control" group, black bar: down-regulated proteins at "5 h zinc vs. 5 h control" group; Bar length showed the corresponding number of regulated proteins.

CHAPER IV DISCUSSION

4.1. Genotypic characterization

The *E. coli* diversity, including various commensal and pathogenic strains, is attributed to the significant variations of genome sizes, ranging from 4.6 Mb to 6.2 Mb, which determines the number of protein-coding genes exhibiting the plasticity of gene composition, thereby resulting in different pathogenicity and physiology [82, 83, 183, 190, 191]. In *E. coli* K-12, the genome size sequenced is about 4.6 Mb containing 4288 protein-coding genes annotated [81]. Its genome size is the shortest among the known *E. coli* genome sizes as well as the number of protein-coding genes predicted, with the exception of *E. coli* BL21 (DE3) that is also a laboratory strain [82, 83].

K-12 as a lab strain has significantly different features compared to *E. coli* strains isolated from a natural host, therefore, in this study we characterized two porcine *E. coli* strains, IMT29408 and IMT8073 correspondingly representing commensal and pathogenic strains to investigate the effects of $ZnCl_2$ on phenotype and protein expression of these two representative strains, as well as comparison with K-12 in phenotype influenced by $ZnCl_2$.

4.1.1. Virulence associated genes (VAGs)

IMT29408 was isolated from a piglet in a zinc feeding trial in control groups and characterized as a major clone among the isolates from various animals in this trial [57]. By screening for virulence associated genes (VAGs) on the whole genome (total genome size: 4,805,298 bp) of IMT29408, it carried 18 VAGs, and none of them resembled any typical type of porcine pathogenic *E. coli*. Most of those VAGs are shown to be common traits in both pathogenic and non-pathogenic strains. Only three of them, *csgA*, *aidA* and *iss* were reported to be specifically associated with increased virulence in InPEC or ExPEC [192-194].

csgA, a curli fimbriae-encoding gene, was demonstrated to be expressed widely among pathogenic and non-pathogenic *E. coli* and even *E. coli* reference strain K-12, but it is involved in disease since biofilm is a virulence feature [192, 195-197]. The *aidA* encoding

adhesin involved in diffuse adhesion has been shown to be common in *E. coli* isolates from clinical healthy swine [198]. Also, it was well demonstrated that virulence associated genes such as *aidA* and *csgA* are present among *E. coli* strains isolated from the intestines of clinically healthy and conventionally reared pigs, indicating that *E. coli* strains harboring some virulence associated genes are a normal part of the intestinal microbiota and not each infection with a pathogen causes disease [94, 99, 199, 200]. Regarding this, one possible role of these virulence associated factors is to colonize intestines as part of a survival mechanism and hence facilitate to increase survival capability in their host animals [84, 94, 198].

The increased serum survival gene *iss* is recognized for playing a role in ExPEC virulence but also frequently present in *E. coli* isolates from the porcine intestine [135, 193]. As for *ibeB* and *ibeC* carried in NMEC that correspondingly encode 50 and 60 kDa membrane proteins involved in invasion of brain endothelial cells, which are in combination with another 50 kDa membrane protein encoded by *ibeA* that is a marker gene in NMEC [201]. Whereas *ibeB* and *ibeC* were found to have homologues, *cusC* (formerly *p77211* encoding copper/silver efflux system) and *eptC* (formerly *yijP* encoding phosphoethanolamine transferase) in K-12, with different functions respectively [192, 201]. It is highly important to notice that there is absence of *ibeA* in IMT29408. Thus, we conclude that both of these genes are rather not responsible for invasion of brain endothelium but for copper/silver efflux and lipid metabolic process. It was also suggested that intestinal commensal *E. coli* could be a reservoir of virulence associated genes for ExPEC [99, 134].

Based on previous observations, we emphasize that drawing conclusions concerning the pathogenicity or non-pathogenicity of particular strains should be not only based on the presence or absence of virulence associated factors, but also include clinical manifestations. In our case, there is a low number of VAGs carried and no clinical diseases displayed in the piglets, thus we consider the intestinal porcine *E. coli* strain IMT29408 as a nonpathogenic (commensal) *E. coli.*

IMT8073 isolated from one four-day old piglet with diarrhea was previously whole genome sequenced (total genome size: 5,149,783 bp) and characterized as aEPEC in our institute [86].

After verification, it harbored Pathogenicity Island LEE (locus of enterocyte effacement, LEE) by demonstrating the presence of *eae*, encoding intimin and used as a marker gene for the presence of the LEE pathogenicity island as well as other LEE-encoded genes like *espA, espD, escD, escC and map*, together with other known genes such as *paa* associated with pathogenesis. However, the EPEC adhesion factor (EAF) plasmid and *stx* genes were absent, which enables us to define this strain as member of the atypical enteropathogenic *E. coli* (aEPEC). The most likely source of this strain might be transmissible through early contact with its maternal sow during nursing or by ingestion of liquid pig manure served as infection source of pathogenic *E. coli* to some extent [202].

4.1.2. Antibiotic resistance genes

Zinc compounds, such as zinc oxide, are used as feed additives to replace the use of antimicrobial growth promoters. One objective of the present study was also to find out whether zinc would influence the expression of antimicrobial resistance genes of *E. coli* IMT29408 and IMT8073 isolated from piglets. Therefore initially the presence of such genes was analyzed.

There were two antibiotic resistance genes (*aadA5* and *tetA*) detected in the genome of porcine *E. coli* IMT29408. Both genes have been reportedly located on plasmids and confer resistance to two classes of antibiotics, namely aminoglycoside and tetracycline [203-205]. Compared to previous resistance patterns of *E. coli* isolates described, it has been found that high rates of antibiotic resistance observed in *E. coli* from pig feces were against tetracyclines, aminopenicillins and aminoglycosides [206], as well as high prevalence observed on resistance to tetracycline, aminoglycosides, ampicillin and sulfamethoxazole among *E coli* isolates from cattle, swine and poultry [207]. Meanwhile, one publication showed that more than 50% *E. coli* isolates were resistant against streptomycin, ampicillin, sulfamethoxazole and tetracycline in genotype and phenotype irrespective of hosts (swine, poultry and bovine) and clinical backgrounds (healthy and sick situation) [208]. Based on these observations, the antibiotic resistances found in our strain are not surprising. Taking into account the current situations about antibiotic resistance in livestock animals, it was assumed that *E. coli* isolates

possessing one or two antibiotic resistant genes would be considered as background level due to widespread use of antimicrobial substances over decades [57, 207]. In addition, since both genes (*aadA5* and *tetA*) were mostly carried on mobile genetic elements, they could easily transfer among *E. coli* by conjugation [209]. Based on the results in genotypic analysis of antimicrobial resistance, IMT29408 was not a multi-resistant strain according to definition of multi-resistance represented by resistance to at least three classes of antibiotics given in literature [210].

E. coli IMT8073 harbored four antibiotic resistant genes encoding for resistance against streptomycin (*strA*/*B*), ampicillin (bla_{TEM_1}) and sulfonamide (*sul2*), belonging to three classes of antimicrobial substances, aminoglycosides, β-Lactam and sulfonamides correspondingly. Combined the characteristics of antimicrobial resistance, IMT8073 was defined as a multi-resistant strain. It has also been more prevalent reportedly to carry linked *strA*/*B* gene pair involved in resistance to streptomycin between *E. coli* isolates from swine [211, 212], as well as the high frequency in resistance against ampicillin and sulfonamide mentioned above in swine feeding. The contribution to this situation is discussed to be mainly due to long-term use of antimicrobial substances in livestock animals, leading to antibiotic resistant bacteria widely distributed in natural environment [211]. Moreover, it has been revealed that there is a correlation of antimicrobial resistance between sows and their offspring, and it was demonstrated that exposure of breeding animals to antibiotics prior to farrowing is able to increase rates of antibiotic resistance in the bacteria of resulting pigs (offspring), in particular during certain phases of production [213]. Thus, it is very likely that this multi-resistant strain originated from sows or environment where sows lived. Although our analyses concentrated on two strains only, in general we would like to mention that the emergence of pathogens with multi-resistance in genotype should be of great concern since it would become a big challenge for clinical treatment with serious limitations in therapeutic strategies.

4.2. Zinc sensitivity

In recent years, in some EU countries like the UK and Spain as well as in USA, zinc compounds, such as zinc oxide, were used in swine industry to combat diarrhea caused by

pathogenic *E. coli* [214]. But no matter what forms of compounds, the free ions are known to be responsible for antibacterial effects in general [215]. Thus, in our study we used zinc chloride, which dissociates easily in LB medium (pH 6.0) to investigate zinc sensitivity among commensal *E. coli* IMT29408, pathogenic *E. coli* IMT8073 and laboratory strain K-12 using MICs test. We observed that the minimal inhibitory concentration (MIC) of zinc chloride on wild-type strains (commensal *E. coli* IMT29408 and aEPEC IMT8073) and K-12 were different, 2 mM against 1 mM (Figure 3-1 and 3-2), suggesting these wild-type strains had a higher capability to defend against zinc stress for survival than that laboratory strain (K-12) under anaerobic conditions. It is very important to emphasize that laboratory K-12 strains of *E. coli* do not represent the wild-type strains which are intestinal commensals or pathogenic ones. Due to the high diversity of *E. coli* genomes sizes (ranging from 4.6 Mb to 6.2 Mb), it is naive to simply rely on published data on K-12 strains [82, 190]. IMT29408 and IMT8073 were not classified as zinc resistant strains, as the MIC_{zinc} values did not reach the tentative cut-off (6 mM) for definition of resistance or susceptibility to zinc according to the previous published values [216].

Zinc sensitivity varied between wild-type strains and K-12. This is easily understandable since K-12 is known and maintained as a reference strain with minimal genetic manipulation lacking the capability to colonize in the intestine and cause disease, possibly this feature influenced the metabolic response to extracellular environments relative to wild-type strains [81, 191]. It seemed common that wild-type bacteria exhibited considerable tolerance to zinc, e.g. more than 1 mM [57, 216], this tolerance or resistance on the one hand was mediated by abundant zinc efflux systems such as ZntA and ZitB decreasing the intracellular zinc concentration [147, 148], as well as by sequestration like ZraP (zinc resistance-associated protein) [217], on the other hand, it was reportedly implicated in antibiotic resistance genes via co-resistance mechanisms (genetic linkage of zinc and antibiotic resistance), e.g. in *Staphylococcus aureus*, its zinc resistance was highly associated with methicillin resistance in genotype [218, 219].

4.3. Bioreactor culture

4.3.1. Growth kinetics of *E. coli* IMT29408 and IMT8073

In previous feeding trials, the expected inhibitory or antibacterial effect of zinc was preferably exerted on pathogenic *E. coli* instead of commensal intestinal bacteria, while which is a great part of gut microbiota. To figure out this effect, we explored the influence of zinc on growth kinetics represented by CFU/ml and OD_{600} in commensal *E. coli* IMT29408 and pathogenic *E. coli* IMT8073.

In IMT29408, supplementation of 1 mM zinc did not significantly influence the cell viability assayed by CFU/ml after continuously monitor from 1 h to 6 h (Figure 3-3), indicating that wild-type *E. coli* IMT29408 had the capability to resist zinc stress and the inhibitory effect of 1 mM zinc on viability of this commensal strain was not obvious. From the growth curves displayed, we found that in the presence of zinc, there was a slight inhibitory effect at 1 h, while after that the curves between zinc treatment and control overlapped and from 5 h on they diverged. At 7 h, the CFU/ml significantly increased in presence of zinc over control. Combined, it is speculated that the bacteria had adapted to this stress environment and could make use of the relative sufficient nutrients for doubling, in comparison with the bacteria cultured in LB medium. Similarly, the bacterial growth rates, as measured by the culture turbidity represented by OD_{600} values between zinc treated and control medium did not have significant differences from 1 h to 7 h, either (Figure 3-3). This observation was in agreement with the finding described in wild-type *E. coli* W3110 cultured in $ZnSO_4$-containing LB medium [220]. During the culture course from 1 h to 6 h, the cell turbidity assayed by OD_{600} values in the presence of zinc was lower than that in the absence of zinc, probably due to the inhibitory effects of zinc on bacterial morphology. At 7 h it reversed, which was consistent with the results of CFU/ml.

As for IMT8073, it displayed no significantly inhibitory influence of 1 mM zinc on the cell viability determined by CFU/ml between both conditions from1 h to 7 h (Figure 3-4). Although the incubated bacterial populations in 1 mM zinc-containing medium were higher than that in control medium but not reaching statistical significance, bacterial cells cultured in

control medium outnumbered that in zinc treatment with a big jump from 1 h to 2 h, indicating that zinc supplemented prevented the bacterial growth to some extent, which could be achieved probably through prolonging the doubling time, 39 min vs. 18 min (Table 3-3), that is, zinc probably exerted on inhibition of key enzymes involved in energy processes, thus preventing the bacterial growth in physiology [221]. Most interestingly, this observation differed from that in commensal strain at the same time period (between 1 h and 2 h); this is due to the differed effects of zinc supplemented on generation time (Table 3-3). After 2 h, the curves displayed an alternate escalation between zinc treatment and control, suggesting that this pathogenic strain adapted the stress condition. In contrast, the bacterial growth rate determined by OD_{600} was significantly reduced after the addition of zinc in comparison to control. It is possible that zinc supplementation could influence bacterial morphology (length or width), as the culture medium was reported to be one determinant for cell size reflected in literature [222]. Our results were consistent with the finding revealed previously that 1 mM zinc acetate decreased significantly the cell densities but not the viability of EPEC cultured in DMEM [159], indicating that there were no bactericidal effects exerted by 1 mM zinc on EPEC.

Therefore, our results provide the evidence that 1 mM zinc does not influence the cell viability of both commensal and pathogenic *E. coli.* It is likely that the inhibition of EPEC virulence caused by 1 mM zinc results from repression of virulence gene expression of EPEC, or changes in bacterial morphology such as damages to the EPEC envelope and/or in physiology, but not from decrease in cell viability [155, 158, 159, 223].

It is possible that the antibacterial effect or bactericidal effect is associated with the zinc compounds used. Although it is known that the zinc ion (Zn^{2+}) may act as the antibacterial effect, leading to zinc chloride, zinc sulfate or zinc acetate widely used in vitro experiment due to its high solubility [215, 220], in-feed zinc oxide or zinc oxide nanoparticles are more popularly fed to animals [224]. It was shown that zinc oxide is partly soluble (about 54% after feed intake) at the stomach acidic pH condition and the rest part is insoluble [216]. Therefore, besides the function initiated by the free zinc ion, the insoluble zinc oxide itself probably has effects on bacterial growth due to the damage to bacterial membrane by generation of H_2O_2

from ZnO surface [225]. The antibacterial activity of zinc oxide nanoparticles, related to the particle size and surface-to-volume ratio, is reportedly driven by inflammation effects such as DNA damage and/or oxidative stress, or by destroying the cell membrane integrity, represented by interacting with membrane lipids, disorganizing the membrane structure and leakage of cytoplasm, which can display the bactericidal effects [223, 226, 227].

4.3.2. Intracellular zinc content measurement

Zinc, as a transition micronutrient, is essential to all forms of life involving numerous cellular processes [4, 140]. However, it is cytotoxic to cells at high levels, thus the amount of intracellular zinc concentration must be tightly controlled in response to various environmental availability of this trace metal [19]. The intracellular zinc amount, including free and associated forms of zinc, varies depending on different culture conditions and is also related to the approach used for measurement, by ratiometric biosensors or inductively coupled plasma mass spectrometry (ICP-MS) [11, 138-140]. Although only a few studies measured the concentration of free zinc or zinc quota at optimum growth conditions in *E. coli* K-12, data about estimating variations of the intracellular amount of zinc over time in wild-type strains under zinc stress and LB medium are still missing [11, 139]. Our present study attempted to investigate how changes of the intracellular zinc content of porcine *E. coli* IMT29408 under exposure to zinc stress -both in control zinc-supplemented LB medium.

In this present study, we cultured the *E. coli* IMT29408 in LB medium using a bioreactor to create constant culture conditions (temperature, pH and atmosphere) resembling the porcine intestinal environment. Our results showed that in *E. coli* IMT29408 cultured in control LB medium, the intracellular zinc content per cell is about 1.1 x 10^5 atoms/cell in average over an incubation period from 1 h to 10 h, with a maximum of 4.5 x 10^5 atoms/cell at 3 h incubation. At this time point, the bacteria are in log phase on the basis of growth curves, thus the bacteria grew actively and divided at a constant, exponential rate. Moreover, at this time point, it is possible that there were more newly synthesized metalloproteins involved in central metabolic pathways or maintaining cellular zinc levels [138, 139]. This resulting zinc content was slightly higher than the measurement (4 x 10^5 atoms/cell) made by Outten et al at midlog

growth of K-12 in LB medium [11]. It is possible that the capacity for "zinc storage" in wild-type strain IMT29408 differed from that in laboratory strain K-12, which could also be considered as an explanation regarding the differences of zinc tolerance in phenotype between IMT29408 and K-12 (Figure 3-1).

By contrast, under addition of 1 mM zinc, the intracellular zinc amount per cell was 8.6 x 10^6 atoms/cell in average from 1 h to 10 h, with a peak amount of 1.2 x 10^7 atoms/cell at 2 h, which was considerably different from the maximum value at 3 h presented in control LB medium, nearly two orders of magnitude. This significant difference also occurred from 4 h to 10 h when comparing identical time points. This result demonstrated that the internal zinc amount varied significantly in association with the extracellular availability of zinc [139]. It can be probably attributed either to the considerable capability of *E. coli* to buffer or store zinc, or to the increased intracellular concentration of free zinc. However, the intracellular free zinc concentration was estimated in *E. coli* K-12 to be between femtomolar range measured in minimal medium and picomolar range measured in 2.5 μM $ZnSO_4$, corresponding to < 1 free zinc ion per cell [11, 139, 140]. Moreover, these available data showed that the increase in intracellular free zinc concentration reaching to picomolar was finished transiently [139, 140].

Taken together, we hypothesized that these increased zinc atoms might be stored through ligands, small molecules, ribosomes and transcriptional factors that function as a zinc buffer as reported in genomic and proteomic analyses [139, 160, 220, 228-230]. Thus, from our results, we concluded that the intracellular zinc content in commensal *E. coli* strain varied significantly influenced by extracellular zinc concentration and even exposure time.

The elevated values at 9 h and 10 h of incubation with zinc, are probably due to that when bacterial growth entered stationary phase, more dead cells existed in the medium while these cells were collected together with viable cells for measuring total zinc concentration. However, the intracellular zinc content per cell was calculated as total zinc concentration dividing by viable CFU numbers instead of total CFU numbers including dead and viable cells, thereby the calculated zinc content per cell is estimated to be higher than the real results showed.

4.4. 2D-DIGE

The protein concentration of each sample in IMT29408 and IMT8073 was measured by Bradford after being extracted from bacteria. In order to evaluate the consistency of protein concentration in each sample from each strain, we took 10 µg proteins from each sample and separated on 1D 12% SDS-PAGE gel. From the bands and resolution of each of the samples from one strain, the proteins among the samples were comparable and reproducible both in IMT29408 as well as in IMT8073. Based on these observations, these protein samples were subsequently analyzed by 2D-DIGE. After labeling all the samples, but prior to performing the second dimensional separation using (0.65 x 20 x 25 cm) SDS-PAGE gels, we carried out 1D 12% SDS-PAGE gel to verify for consistency and efficiency of the labeled samples so as to ensure feasibility for further performance. According to the fluorescent signals of protein bands and resolution (Figure 3-8), the labeling worked efficiently. The resulting 2D-DIGE images from big SDS-PAGE gels were comparable with high reproducible protein spots.

4.5. Quantitative analysis

There were 544 versus 413 protein spots detected in total in IMT29408 and IMT8073, respectively. The resulting difference is probably attributable to the number of protein-coding genes or technical problems (software limitation for spots with low intensity or spots editing errors). In contrast, the number of differentially expressed spots in IMT29408 was lower than that in IMT8073, 172 vs. 200. Taken together, the phenomenon can be due to the fact that both analyzed strains vary in genomic contents. Thus the commensal strain had more regulated genes encoding proteins or enzymes involved in metabolic pathways in response to the stimuli they encounter compared to the pathogenic aEPEC strain [83].

4.6. Protein identification

Some differentially expressed spots with low expression concentrations were able to be recognized and detected by DECODON software analysis, while it had the visible limitations for excision of these protein spots from SDS-PAGE gels stained with CBB, though the sensitivity of protein detection down to 30 ng of CBB was achieved [231]. Therefore,

obviously a discrepancy exists between software analyses and visible recognition of spots stained with CBB, leading to unsuccessful excision of some differentially expressed spots or even to some technical errors during cutting. Several protein spots failed identification, which was attributed to the lower concentration of extracted peptides via in-gel digestion using trypsin, thus generating insufficient spectra for protein identification. Several proteins were identified in more than one location on the gels, such as Pgk and TpiA in IMT29408 as well as TnaA and AtpD in IMT8073, representing protein isoforms with different post-translational modifications [232].

4.6.1. Commensal *E. coli* IMT29408

With the known benefits from zinc supplementation by acting not only on the host but also on the bacteria, most data obtained by previous groups mainly focused on genomic and proteomic level of laboratory strains of *E. coli* induced by zinc [153, 161, 228]. It made the results difficult to extrapolate to wild-type strains which are professional intestinal commensal *E. coli*. Thus, our purpose in this study was to get insight into how commensal *E. coli* respond to zinc stress at proteomic level. We identified a greater number of differentially expressed proteins and most of them have not previously been implicated in responding to zinc stress compared to the previous findings focusing on *E. coli* K-12 using 2D-gel for separation [160, 161]. Comparatively, there were only three proteins (RbsB, Tsf and Mdh) that were identical to our results [160, 161]. The resulting discrepancy was presumably due to (1) the higher sensitivity of 2D-DIGE combined with DECONDON software analysis we used, and (2) the different number of genes that encode proteins involved in response to zinc stress between commensal strain and K-12 [81, 83]. Among the identified proteins with significance of expression (more than 1.5-fold change, $p<0.05$), 2 h exposure to zinc resulted in up-regulated proteins involvement in biosynthetic process (FabF) and stress response (DegP) but down-regulation of more proteins involved in translation (Tsf), glycolysis (Pgk), tricarboxylic acid cycle (Mdh), transcription (RpoA), transport (ManX and RbsB), and oxidation-reduction process (YhdH). Whereas, after 5 h treated with zinc, IMT29408 mainly enhanced the expression of proteins involved in translation (RplD), glycolysis (TpiA), ATPase activity (YchF) and transport (Tsx). Continuous zinc treatment from 2 h to 5 h mainly influenced the

expression of proteins taking part in translation (ArgS and AsnS up-regulated while FusA, Tuf, RplJ and SerS down-regulated) and biosynthetic processes (FabI up-regulated while AccA, FabF and GuaB down-regulated). It also led to the down-regulation of proteins associated with membrane permeability (OmpX and OmpC), oxidation-reduction process (AdhE and HyaB) and glycolysis (Pyk). Taken together, it was suggested that commensal *E. coli* adapted to zinc stress by up-regulation of proteins involved in translation, transport and energy-generated activities such as ATPase activity and glycolysis as one survival strategy, while persistent incubation with zinc resulted in reduced cell permeability, indicating that this strain adapted to the changes in outer membrane communication to zinc stress.

It is known that when encountered extracellular environments, bacteria are through regulating synthesis of defensive proteins against these detrimental effects [233]. A number of proteins, grouped into oxidative stress response proteins and general stress response proteins, have been identified and shown to play a role in protecting cells from different stresses [234, 235]. In IMT29408, DegP (HtrA), a *rpoE*-dependent protein, was up-regulated significantly upon exposure to zinc at both 2 h and 5 h respectively, and it participated in cell envelope response by degradation of unfolded or misassembled periplasmic proteins [178, 236]. The bacterial membrane interacting with excess metal may contribute to denaturation of cell surface proteins, which are repaired by refolding systems or degraded by protease systems. This assumption was supported by the fact that *rpoE* and *ropE*-dependent genes like *degP* involved in envelope stress response were significantly up-regulated after 5 min exposure to 0.5 mM $ZnCl_2$ in wild-type *E. coli* W3110 at the transcriptional level [220]. Likewise, RplD which was increased is primarily one ribosomal component of the translational machinery. It was found that the increase of RplD might elevate the production of stress-induced proteins by inhibiting RNase E-dependent decay, enhancing bacterial adaptation to adverse environments [237]. While AhpC, as a scavenger of endogenous H_2O_2 during oxidative stress, was down-regulated after 5 h treated with zinc compared to control, this might be a general consequence [238]. Since it was possible that during the stationary phase the clearance of H_2O_2 was achieved by KatE or KatG, or at this phase the commensal strain had adapted to the high extracellular zinc [239]. This situation was in agreement with the response of *E. coli* to metallic copper surfaces

[240]. In addition, GroES was increased 3.3-fold higher in “2 h zinc vs. 5 h zinc only” group. GroES, a general stress response protein and co-chaperonin of GroL (Hsp60), is required for cell survival under adverse conditions [241]. Taken together, it was proposed that these stress-responding proteins noted in our study were involved in resistance against zinc stress of the commensal strain.

In theory, zinc stress triggers the expression of proteins that are involved in zinc transportation and/or sequestration, facilitating bacteria to maintain zinc homeostasis and survive under the stress condition [3]. Therefore, these proteins in *E. coli* encountering zinc stress should be induced and thus detected by proteomic analyses. However, in our proteomic study on the response of commensal *E. coli* to zinc stress, none of the known zinc transporters, especially efflux transporters, e.g. ZntA and ZitB, could be detected. The same was true for BasR and BasS which are involved in detoxification of zinc in *E. coli* [150] as well as for the zinc resistance-associated protein ZraP [217], though we used a p*I* range of 3-10 in order to attempt to cover any possible proteins and quantified the protein expression on 2D-DIGE by DECODON Delta2D with higher sensitivity and accuracy. These zinc-transporting proteins could not also be detected in *E. coli* laboratory strains using a p*I* range of 4-7 separated on 2D gels [160, 161]. Based on these observations, the possible potential explanations for this phenomenon were that (1) these zinc transporting associated proteins are localized on the cell membrane with insoluble properties, thus they were not extracted from the bacterial lysate; (2) the expression levels did not reach to the cut-off value of 1.5-fold after 2 h or 5 h challenged with zinc using the Dalt2D analysis software; (3) it was possible that the regulation of zinc transporter synthesis for the adaptation to zinc stress occurred in a rapid way, since synthesis of zinc transporters is regulated by the intracellular free zinc concentration [140]. It was demonstrated that exposure to 2.5 μM $ZnSO_4$ leads to a transient increase in intracellular zinc concentration [140], which was supported by one previous finding at transcription level that *zntA* expression increases at 5 min after addition of 0.5 mM $ZnCl_2$ but decreases at 30 min [220]; (4) these protein spots could not be successfully excised from the SDS-PAGE gel due to the visible limitations for recognition of these protein spots with lower intensity; (5) alternatively, these zinc efflux transporters failed to be identified because of insufficient

spectra generated by MALDI-TOF MS/MS. However, in previous studies at the transcriptional level, *zntA, basR* and *basS* were up-regulated in response to 0.2 mM $ZnSO_4$ by microarray analyses [220, 228]. It was possible that there was no a direct correlation between transcript levels and protein level [160]. Combined all, these indicated that *E. coli* adapted to zinc stress by multiplex mechanisms including not only transporting systems, but also intracellular zinc buffer and/or metabolic activities probably.

Over the recent years with intense exposure of farmed animals to this trace element that was subsequently released to the environment through animal liquid manure, a resistance-driven effect reportedly occurred in microorganisms among animal host and environment [57, 182, 242, 243]. So far, there is no sufficient evidence of interaction between heavy metals and antimicrobial resistance with bacteria, except that one mechanism revealed is due to co-selection of metal and antibiotic resistance [218, 244]. In this study, we expected to figure out differentially expressed proteins that were associated with antibiotic resistance under zinc stress. Increased duration of zinc treatment (from 2 h to 5 h) resulted in the down-regulation of two porins, OmpC and OmpX. The reduction of OmpC was corroborated in several publications to be involved in resistance against certain hydrophilic antibiotics such as ciprofloxacin and norfloxacin [178, 245-247]. Furthermore, the deletion of *ompX* led to an increased tolerance against hydrophobic antimicrobial compounds such as novobiocin and gentamicin [248]. As outer membrane permeability of gram-negative bacteria like *E. coli* are controlled by porin proteins and diffusion channels, therefore down-regulation of which can lead to the alteration of permeability that is one mechanism responsible for antibiotic resistance [176, 178, 179, 249, 250]. Our data indicated that prolonged exposure to zinc treatment may cause a decrease of OmpC and OmpX expression. This may result in the emergence of antibiotic resistance [178]. Therefore, the results in the present study arose the hypothesis that long-term or persistent use of high concentration of zinc could induce the reduced expression of outer membrane porins, thus leading to the increased emergence of antibiotic resistance cases.

In addition, we assumed that the role of increased DegP at both 2 h and 5 h in this commensal strain may participate in antibiotic resistance through degrading misassembled porins

accumulated within the periplasm, as shown by Viveiros et al. [178, 251, 252]. In their study, the expression of DegP was increased in K-12 when resistance to tetracycline had increased to 10 mg/l from 4 mg/l, together with down-regulation of OmpC and OmpF [178].

4.6.2. Pathogenic *E. coli* IMT8073 (aEPEC)

To the best of our knowledge, this is the first proteomic study about pathogenic *E. coli* in response to zinc. In our study, we found that at 2 h, zinc treatment only elevated the expression of two proteins involved in oxidative stress (KatE and ClpB). After 5 h of exposure to zinc, this aEPEC strain up-regulated more proteins taking part in translation (AsnS and Tsf), ATPase activity (AtpA and AtpD), anaerobic respiration (HybC), biosynthetic process (FabF, PyrG and AccC), metabolic process (TnaA and AspA), and stress response (DegP), compared to the down-regulated ones participating in transcription (NusA) and metabolic processes (Udp, PflB, MinD, CadA and NanA). These findings indicated that up-regulation of proteins associated with oxidative stress, energy generation, translation, and biosynthesis might be a survival mechanism for this pathogenic strain under zinc stress. Continuous zinc treatment from 2 h to 5 h mainly influenced the proteins related to translation (PrfB and LysS up-regulated while Tuf, GlyS, TypA, RplI and GlnS down-regulated), metabolic processes (GlpQ, GlnA and AspA up-regulated while FbaA, Pta, TdcE, PflB, MinD, Gnd, CadA, PspE and EutD down-regulated), biosynthetic processes (PdxJ up-regulated while FabF, GlmS and HemX down-regulated) and transcription (Rho, RpoA and NusA down-regulated). The down-regulation of three proteins (YchF, FumA and Pyk) associated with energy-generated activities, was probably compensated by up-regulation of HybC involved in anaerobic respiration. The data suggested that this aEPEC *E. coli* responded to zinc stress at the translational level in a similar time-dependent adaptation as was shown for the commensal strain and K-12 [161]. Also, these results improved the basic understanding of pathogenic *E. coli* responding to zinc stress at proteomic level.

Zinc is widely introduced as an alternative additive in food-producing animal feeding due to the antibacterial effects of zinc on pathogenic bacteria. It was demonstrated that high concentrations of zinc could not only inhibit virulence gene expression of enteric pathogens

including EPEC, EAEC, and STEC as well as *Campylobacter jejuni*, but also ameliorate intestinal inflammation of host tissues [53, 154-156, 233]. By analysis of zinc-modulated stress proteins in pathogenic *E. coli* IMT8073, it was shown that 2 h exposure to 1 mM zinc compared to control resulted in the increase of two proteins, KatE and ClpB, both of which were the only differentially expressed protein spots at 2 h displayed in this pathogenic strain. It was shown that the oxidative stress protein KatE, a catalase HPII, is required for bacterial survival in many stress conditions, together with general stress chaperone protein ClpB [234, 235, 253]. In addition to that, GroES that up-regulated from 2 h zinc to 5 h zinc, is required for cell survival under adverse conditions [241]. After 5 h exposure to 1 mM zinc in comparison to control, DegP (HrtA) was up-regulation (2-fold), and is *rpoE*-dependent [158, 220]. In previous reports, 0.3 mM zinc acetate induced up-regulation of envelope stress markers (*degP, dsbA* and *rpoE*) in EPEC at the transcriptional level, indicative of the activation of envelope stress response, and it was demonstrated that the zinc-driven envelope stress pathway mediated the reduction of EPEC virulence, e.g. downregulation of type III secretion [158, 159, 254]. Thus, we speculated that 1 mM zinc stress probably resulted in occurrence of oxidative stress and envelope stress in EPEC. It was likely that one of the mechanisms of antibacterial effect of zinc was by increasing in oxidative stress level of bacteria cells. This was also described in *Campylobacter jejuni* [233].

According to our results, we found that continuous incubation of zinc (from 2 h to 5 h) resulted in a decrease of TypA (BipA) in aEPEC IMT8073. TypA, a member of GTP-binding protein superfamily exhibiting GTPase activity that is involved in cell growth [255], was characterized in pathogenic bacteria such as EPEC and *Salmonella enterica enterica* Serovar Typhimurium [256-258]. It was found that TypA, as a regulatory protein, played a role in the pathogenesis of EPEC and the TypA mutation in EPEC led to a declined ability to form attaching and effacing lesions in infected epithelial cells [257, 259]. Combining these observations, we proposed that continuous incubation with zinc (from 2 h to 5 h) reduced the expression of TypA, and this reduction influenced the interaction of TypA with its global regulatory networks and its phosphorylation, thus affecting the virulence of EPEC [256, 260]. This should be further elucidated. Additionally, our results revealed that NanA

(N-acetylneuraminate lyase) was down-regulated after 5 h exposure to zinc compared to control medium. NanA is involved in sialic acid catabolic process in *E. coli* [261]. It was shown that intestinal colonization and persistence of *E. coli* K-12 in mice was affected slightly when *nanA* was deleted [262]. Taken together, it was indicated that the antibacterial effects of zinc on EPEC were likely achieved by a combination of multiple physiological mechanisms such as oxidative stress, envelope stress and metabolic influences, which might be a possible explanation for previous findings revealed that zinc could reduce the virulence of EPEC.

CadA (lysine decarboxylase) responsible for metabolizing lysine, was down-regulated with 6.2-fold change after 5 h exposure to zinc compared with control as well as 4.1-fold down-regulation from 2 h to 5 h zinc treatment, also functioning in protection of *E. coli* K-12 from fermentation acids during starvation of phosphate [263]. Previous studies proposed that *cadA* is an antivirulence gene in EIEC and *Shigella* spp. [264]. However, this was only due to toxin production of these bacteria. It was demonstrated that EHEC with *cadA*-mutant resulted in increased adherence to tissue-cultured cells and intestinal colonization [265, 266]. The reason was that CadA is as negative regulator of the EHEC adherence through mediating the expression of outer membrane adhesin, intimin encoded by *eae* gene [265]. By contrast, in our case, CadA was down-regulated, and it seemed that zinc treatment could probably increase the expression of intimin, on the other hand, we hypothesized that this resulting increase probably cost much energy for pathogenic *E. coli*, thus exhibiting adverse effects to pathogens. Further elucidation remains to be performed.

4.6.3. Comparative proteomic analysis between IMT29408 and IMT8073

Among the differentially expressed proteins identified between IMT29408 and IMT8073, several the same proteins between both strains including DegP, FabF, TnaA, GroES, Tuf, AdhE, Pyk and PflB, have consistent expression patterns (Table 3-8), indicating that these proteins were required for survival of both *E. coli* strains under zinc stress. In comparison of identified proteins between commensal strain IMT29408 and pathogenic strain IMT8073 at 2 h, we observed that zinc treatment resulted in up-regulated proteins only responsible for

oxidative stress in the aEPEC strain, while proteins associated with multiple functions were regulated by zinc in the commensal strain, suggesting that the commensal *E. coli* had an advantage for adaption to zinc stress by activation or repression of protein-encoded genes expression [267]. However, at 5 h both strains had a similar expression pattern, mainly by up-regulation of proteins involved in stress defense, translation and energy generation (ATP synthesis, glycolysis, and anaerobic respiration), which were likely to be the survival strategy for both strains in the presence of zinc.

For continuous zinc treatment from 2 h to 5 h, the main differences were that 1) there were more proteins associated with metabolic processes influenced by zinc in pathogenic strain than that in commensal strain, twelve against one; 2) zinc treatment led to reduced cell permeability in commensal strain but not in pathogenic strain. This could be either interpreted as commensal strain had developed the advantages against the environmental conditions which it experienced but for pathogenic strain which should be via utilization of more metabolic activities to adaptation of the adverse conditions, or due to the different numbers of genes that code proteins involved in physiological activities between both strains [82, 83, 268].

4.6.4. Future work

Based on the results presented in this study, we expect to perform the following studies in the future.

1. Investigate intracellular zinc content in pathogenic strain as described above in commensal strain to compare the zinc-stored capabilities in pathogenic strain with commensal strain.

2. Select several commensal strains from our collection isolated from porcine intestine and explore the antibiotic resistance patterns of these strains after incubation with zinc by MIC compared to the results observed before zinc treatment, in order to confirm if zinc treatment leads to an increased resistance against certain antibiotics in phenotype.

3. Confirm if outer membrane proteins such as OmpC, OmpF and OmpX are down-regulated by Western blot after continuous zinc treatment and detect if AcrAB effulx system proteins

(AcrA, AcrB and TolC) involved in antibiotic resistance were regulated by zinc exposure.

4. Verify whether continuous zinc treatment induces down-regulation of TypA, NanA and CadA by Western blot and investigate the effects of zinc on adherence of EPEC to cultured epithelial cells in vitro.

SUMMARY

Effects of zinc on protein expression of porcine commensal and pathogenic *Escherichia coli* (*E. coli*)

Zinc is a micronutrient required for numerous cellular functions in all living organisms. In recent years, zinc as an alternative additive is increasingly used in farmed animals to enhance growth performance, as it is able to prevent or reduce the duration and severity of diarrhea caused by some pathogens, especially pathogenic *E. coli*. It has been reported that zinc supplementation also leads to an increased rate of antibiotic resistance in commensal *E. coli*. However, until now the underlying mechanisms of these effects driven by zinc exposure have remained unclear. In our studies, we characterized two *E. coli* strains, commensal strain IMT29408 and pathogenic strain IMT8073 (aEPEC) defined by genotypic analysis, as representatives of porcine intestinal *E. coli* to investigate zinc sensitivity, the effects of zinc on growth rates, intracellular zinc content of commensal strain in vitro and protein expression differences induced by zinc. Since there is only limited available knowledge about laboratory strains (K-12) instead of wild-type strains that are highly different in gene content and consequent physiological properties compared to lab strains.

In the present study, the bacteria were cultured in LB medium using a bioreactor under anaerobic condition with or without 1 mM zinc chloride determined on the basis of minimal inhibitory concentration test. There were no significantly inhibitory influences of zinc on cell viability represented by CFU per milliliter and cell turbidity represented by OD_{600} values from 1 h to 7 h in commensal strain IMT29408. By contrast, although the cell viability of aEPEC IMT8073 was not significantly decreased by zinc, the bacteria turbidities were reduced by zinc treatment from 1 h to 7 h. These indicated that the inhibition of aEPEC virulence was likely not induced by the reduction in bacterial viability, but rather by changes in cell physiology.

In the commensal strain IMT29408, we investigated how the intracellular zinc content changes in the presence or absence of 1 mM zinc over time. The results showed that from 0 h to 10 h of incubation, the intracellular zinc content was about 8.6 x 10^6 atoms/cell in average with a maximum of 1.2 x 10^7 atoms/cell at 2 h in the presence of zinc, while cultured in the

control (LB medium), the average value was about 1.1 x 10^5 atoms/cell with a peak amount of 4.5 x 10^5 atoms/cell at 3 h. The results suggested that the intracellular zinc content in commensal *E. coli* varies significantly with the changes in environmental zinc availability and exposure time.

Based on the growth rates and intracellular zinc content curves over time, bacterial samples were collected at 2 h and 5 h time points for proteomic analysis to investigate the differences of protein expression between zinc treatment and control. In commensal *E. coli* IMT29408, proteomic analysis using 2D-DIGE revealed 544 protein spots in total. We identified two differentially expressed (>1.5-fold change, $p<0.05$ (student's *t* test)) proteins up-regulated (DegP and FabF) and seven proteins down-regulated (ManX, Mdh, Pgk, RbsB, RpoA, Tsf and YhdH) at 2 h of incubation with zinc using MALDI-TOF MS/MS. At 5 h of incubation, there were seven up-regulated (DegP, FabF, RplD, TnaA, TpiA, Tsx and YchF) and three down-regulated (AhpC, RffG and PflB) proteins identified. Most of them have not previously been implicated in responding to zinc compared with the available data about *E. coli* K-12, which are mainly involved in translation, stress defense, glycolysis and transport. We also identified the differentially expressed proteins only influenced by continuous zinc treatment (from 2 h to 5 h), six up-regulated and fourteen down-regulated. Among these, two porins OmpC and OmpX were down-regulated, which suggested that persistent zinc treatment could result in reduced outer membrane permeability. These data give evidence that reduced outer membrane permeability is a possible explanation for the zinc-induced antibiotic resistance.

In pathogenic *E. coli* IMT8073, there were 413 protein spots detected by 2D-DIGE. Out of them, at 2 h of incubation we identified two up-regulated proteins (KatE and ClpB), while at 5 h, there were eleven up-regulated (AccC, AsnS, AspA, AtpA, AtpD, DegP, FabF, HybC, PyrG, TnaA and Tsf) and six down-regulated (CadA, MinD, NanA, NusA, PflB and Udp) proteins identified. From these identified proteins, we concluded that pathogenic *E. coli* adapted to zinc stress by up-regulation of proteins associated with oxidative stress defense, translation, and energy generation (ATP synthesis, glycolysis, and anaerobic respiration) as a survival strategy. We also identified eight up-regulated and twenty-six down-regulated proteins only influenced by zinc when comparing 2 h to 5 h of incubation. Most of these

proteins were involved in translation (PrfB and LysS up-regulated while Tuf, GlyS, TypA, RplI and GlnS down-regulated), metabolic processes (GlpQ, GlnA and AspA up-regulated while FbaA, Pta, TdcE, PflB, MinD, Gnd, CadA, PspE and EutD down-regulated) and biosynthetic processes (PdxJ up-regulated while FabF, GlmS and HemX down-regulated). Of these proteins, TypA, a regulatory protein was down-regulated and this suggested that persistent zinc treatment probably influenced EPEC virulence, as *typA*-mutants of EPEC showed declined adherence to cultured cells [257, 259]. Based on these results, it was indicated that the antibacterial effects of zinc on EPEC are likely achieved by a combination of multiple physiological mechanisms such as oxidative stress, envelope stress and metabolic influences, which might be a possible explanation for previous findings that zinc treatment reduces virulence of EPEC [158, 159].

Comparatively, the differentially expressed proteins influenced by zinc were mostly different among the wild-type *E. coli* strains (commensal and pathogenic) in the present study as well as the *E. coli* K-12 described previously. This indicated that the metabolic strategies for adaptation of zinc stress vary among these strains, which is probably due to the different number of protein-coding genes involved in physiological activities.

ZUSAMMENFASSUNG

Die Wirkung von Zink auf die Proteinexpression von porzinen kommensalen und pathogenen *Escherichia coli (E. coli)*

Zink ist ein Spurenelement, das für zahlreiche zelluläre Funktionen in allen lebenden Organismen benötigt wird. In den letzten Jahren nahm die Verwendung von Zink als Alternative zu antibiotischen Leistungsfördereru als Futterzusatz in der landwirtschaftlichen Tierhaltung zu, um vor allem das Wachstum der Tiere zu fördern. Es wird außerdem angenommen, dass Durchfallerkrankungen, die durch Pathogene und zwar speziell durch *E. coli* verursacht warden, durch die Zugabe von Zink verhindert oder die Dauer und der schwere Verlauf abgemildert werden können. Andererseits wurde berichtet, dass die Zugabe von Zink zu einem höheren Auftreten von Antibiotikaresistenzen in kommensalen *E. coli* führt. Bislang sind die dafür verantwortlichen Mechanismen unklar. In unseren Untersuchungen haben wir zwei *E. coli* Stämme charakterisiert, den kommensalen Stamm IMT29408 und den pathogenen Stamm IMT8073 (aEPEC), welche durch eine genotypische Analyse definiert wurden. Die Stämme wurden repräsentativ für porzine intestinale *E. coli* ausgewählt um die Zinksensivität, die Effekte von Zink auf die *E. coli* Wachstumsrate, die intrazelluläre Zinkkonzentration und die Unterschiede in der Proteinexpression, welche durch Zink induziert werden, zu untersuchen. Bisherige Zink-abhängige Proteinexpressionsstudien konzentrierten sich auf *E. coli* K-12 Laborstämme. Diese Stämme unterscheiden sich jedoch enorm von Wildtyp-Stämmen - sowohl bezüglich Genomgröße als auch der dadurch bedingten physiologischen Eigenschaften. Daher können die bekannten K-12- abhängigen Ergebnisse nicht auf Wildtyp-Stämme übertragen werden.

In der aktuellen Studie wurden die *E. coli* Stämme in LB Medium mit/ohne 1mM Zinkchlorid anaerob in einem Biorektor kultiviert. Die verwendete Konzentration wurde mittels minimalen Hemmkonzentrationstests bestimmt. Es gab keine signifikanten inhibitorischen Einflüsse von Zink auf die Zellvitalität (KBE pro Milliliter) und Zelldichte (OD600nm Werte von 1-7 Stunden) im kommensalen Stamm IMT29408. Die Zellvitalität des aEPEC Stamms wurde auch nicht signifikant durch Zink beeinflusst. Jedoch war die Bakteriendichte

signifikant durch die Kultivierung mit Zink reduziert. Dies könnte darauf hinweisen, dass die Virulenz von aEPEC weder durch die Reduktion der Vitalität noch durch Veränderungen in der Zellmorphologie oder Physiologie inhibiert wird.

Die Untersuchung des intrazellulären Zinkgehalts in An- und Abwesenheit von Zink im kommensalen Stamm IMT29408 ergab, dass während der Kultivierung mit Zink der intrazelluläre Zinkgehalt durchschnittlich bei 8,6 x 10^6 Atomen/Zelle lag. Er erreichte sein Maximum nach zwei Stunden mit 1,2 x 10^7 Atomen/Zelle. Bei der Kultivierung ohne Zink im reinen LB-Medium lag der Mittelwert bei 1,1 x 10^5 Atomen/Zelle mit einem Höchstwert von 4,5 x 10^5 Atomen/Zelle. Die Ergebnisse weisen darauf hin, dass die intrazelluläre Zinkkonzentration in kommensalen *E. coli* signifikant mit den Veränderungen in der Verfügbarkeit von Zink in der Umwelt und dem Zeitraum, dem es Zink ausgesetzt ist, variiert.

Basierend auf den zeitabhängigen Wachstumsraten und der intrazellulären Zinkkonzentration wurden Proben nach 2 und 5 Stunden für die Proteomanalyse gewonnen, um Unterschiede in der Proteinexpression zwischen den Gruppen der Zinkbehandlung und Kontrolle zu messen. Die Proteomanalyse des kommensalen *E. coli* IMT29408 mit 2D-DIGE und MALDI-TOF MS/MS zeigte 544 Proteinspots auf. Darunter konnten wir Proteine identifizieren, die unterschiedlich exprimiert werden (>1.5-fold change, $p<0.05$ (student's t test)). Es gab zwei Proteine, die nach zwei Stunden induziert (DegP und FabF) und sieben Proteine, die reprimiert waren (ManX, Mdh, Pgk, RbsB, RpoA, Tsf und YhdH). Nach einer Inkubationszeit von fünf Stunden waren sieben Proteine induziert (DegP, FabF, RplD, TnaA, TpiA, Tsx und YchF) und drei reprimiert (AhpC, RffG und PflB). Die meisten dieser Proteine sind bislang nicht bekannt dafür auf Zink zu reagieren, beruhend auf den Daten mit *E. coli* K-12. Die genannten Proteine sind hauptsächlich involviert in der Translation, der Stessantwort, der Glykolyse und dem Transport. Wir konnten ebenfalls Proteine identifizieren, die nur durch Zink beeinflusst wurden (nach zwei und fünf Stunden). Es waren sechs induzierte und 14 reprimierte Proteine. Unter diesen waren auch die zwei Porine OmpC und OmpX reprimiert. Dies führt uns zu der Annahme, dass eine andauernde Zinkbehandlung in einer reduzierten äußeren Membranpermeabilität resultiert. Diese Daten könnten somit einen Hinweis für einen möglichen Mechanismus für die zinkinduzierte Antibiotikaresistenz liefern.

Für den aEPEC Stamm IMT8073 konnten insgesamt 413 Proteinspots durch die 2D-DIGE detektiert werden. Nach zwei Stunden Inkubation waren zwei Proteine induziert (KatE und ClpB), während nach fünf Stunden elf Proteine induziert (AccC, AsnS, AspA, AtpA, AtpD, DegP, FabF, HybC, PyrG, TnaA und Tsf) und sechs reprimiert waren (CadA, MinD, NanA, NusA, PflB und Udp). Die Identifizierung dieser Proteine lässt die Annahme zu, dass pathogene *E. coli* sich an die hohe Zinkkonzentration adaptieren können durch Hochregulierung von Proteinen, die mit oxidativer Stressantwort, Translation und Energiegeneration (ATP Synthese, Glykolyse und anaerobische Atmung) assoziiert sind und dies als eine Überlebensstrategie nutzen. Zudem wurden acht induzierte und 26 reprimierte Proteine durch den Einfluss von Zink identifiziert (nach zwei und fünf Stunden Kultivierung mit Zink). Die meisten von diesen Proteinen sind in der Translation (PrfB und LysS induziert während Tuf, GlyS, TypA, RplI und GlnS reprimiert), metabolischen Prozessen (GlpQ, GlnA und AspA induziert während FbaA, Pta, TdcE, PflB, MinD, Gnd, CadA, PspE und EutD reprimiert) und biosynthetischen Prozessen involviert (PdxJ induziert während FabF, GlmS und HemX reprimiert). Die Daten weisen darauf hin, dass TypA an der Adhärenz von EPEC beteiligt sind [257, 259]. Daher könnte die Repression von TypA durch Zink von Bedeutung für die Virulenz sein. Basierend auf diesen Ergebnissen nehmen wir an, dass der mögliche antibakterielle Effekt von Zink auf aEPEC durch die Kombination von mehreren physiologischen Mechanismen erreicht wird, wie zum Beispiel oxidativen Stress und metabolischen Einflüssen. Dies ist eine mögliche Erklärung für den in früheren Studien festgestellten Effekt, dass Zink die Virulenz von EPEC reduziert [158, 159].

Die durch Zink verursachte unterschiedliche Proteinexpression war besonders deutlich bei den *E. coli*-Stämmen des Wildtypes (kommensal und pathogen) in dieser Studie, sowohl als auch in dem vorher beschriebenen *E. coli* K-12. Das weist darauf hin, dass metabolische Strategien für die Adaptation bei Zinkstress zwischen den verschiedenen Stämmen variieren, möglicherweise durch die unterschiedliche Anzahl an Protein-kodierenden Genen, die in physiologischen Aktivitäten involviert sind.

RERERENCES

1. McCall KA, Huang C, Fierke CA: Function and mechanism of zinc metalloenzymes. *The Journal of nutrition* 2000, 130(5S Suppl):1437S-1446S.

2. Todd WR, Elvehjem CA, Hart EB: Zinc in the nutrition of the rat. *American Journal of Physiology* 1934, 107:145-156.

3. Blindauer CA: Advances in the molecular understanding of biological zinc transport. *Chemical communications* 2015, 51(22):4544-4563.

4. Coleman JE: Zinc enzymes. *Current opinion in chemical biology* 1998, 2(2):222-234.

5. Maret W: Zinc biochemistry: from a single zinc enzyme to a key element of life. *Advances in nutrition* 2013, 4(1):82-91.

6. Vallee BL, Falchuk KH: The biochemical basis of zinc physiology. *Physiological reviews* 1993, 73(1):79-118.

7. Vallee BL, Auld DS: Functional zinc-binding motifs in enzymes and DNA-binding proteins. *Faraday discussions* 1992(93):47-65.

8. Porcheron G, Garenaux A, Proulx J, Sabri M, Dozois CM: Iron, copper, zinc, and manganese transport and regulation in pathogenic Enterobacteria: correlations between strains, site of infection and the relative importance of the different metal transport systems for virulence. *Frontiers in cellular and infection microbiology* 2013, 3:90.

9. Andreini C, Banci L, Bertini I, Rosato A: Counting the zinc-proteins encoded in the human genome. *Journal of proteome research* 2006, 5(1):196-201.

10. McCance RA, Widdowson EM: The absorption and excretion of zinc. *The Biochemical journal* 1942, 36(7-9):692-696.

11. Outten CE, O'Halloran TV: Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* 2001, 292(5526):2488-2492.

12. Vallee BL, Coleman JE, Auld DS: Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proceedings of the National Academy of Sciences of the United States of America* 1991, 88(3):999-1003.

13. Williams RJ: Zinc: what is its role in biology? *Endeavour* 1984, 8(2):65-70.

14. Hood MI, Skaar EP: Nutritional immunity: transition metals at the pathogen-host interface. *Nature reviews Microbiology* 2012, 10(8):525-537.

15. Vallee BL, Auld DS: Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* 1990, 29(24):5647-5659.

16. Prasad AS: Impact of the discovery of human zinc deficiency on health. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements* 2014, 28(4):357-363.

17. Katayama A, Tsujii A, Wada A, Nishino T, Ishihama A: Systematic search for zinc-binding proteins in *Escherichia coli. European journal of biochemistry / FEBS* 2002,

269(9):2403-2413.

18. Blindauer CA, Harrison MD, Robinson AK, Parkinson JA, Bowness PW, Sadler PJ, Robinson NJ: Multiple bacteria encode metallothioneins and SmtA-like zinc fingers. *Molecular microbiology* 2002, 45(5):1421-1432.

19. Cerasi M, Ammendola S, Battistoni A: Competition for zinc binding in the host-pathogen interaction. *Frontiers in cellular and infection microbiology* 2013, 3:108.

20. Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM: Metal ions in biological catalysis: from enzyme databases to general principles. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2008, 13(8):1205-1218.

21. Riordan JF: Biochemistry of zinc. *The Medical clinics of North America* 1976, 60(4):661-674.

22. Yao S, Flight RM, Rouchka EC, Moseley HN: A less-biased analysis of metalloproteins reveals novel zinc coordination geometries. *Proteins* 2015, 83(8):1470-1487.

23. Bode W, Gomis-Ruth FX, Huber R, Zwilling R, Stocker W: Structure of astacin and implications for activation of astacins and zinc-ligation of collagenases. *Nature* 1992, 358(6382):164-167.

24. Gregory DS, Martin AC, Cheetham JC, Rees AR: The prediction and characterization of metal binding sites in proteins. *Protein engineering* 1993, 6(1):29-35.

25. Kim EE, Wyckoff HW: Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. *Journal of molecular biology* 1991, 218(2):449-464.

26. Vallee BL, Auld DS: Active-site zinc ligands and activated H2O of zinc enzymes. *Proceedings of the National Academy of Sciences of the United States of America* 1990, 87(1):220-224.

27. Vallee BL, Auld DS: Cocatalytic zinc motifs in enzyme catalysis. *Proceedings of the National Academy of Sciences of the United States of America* 1993, 90(7):2715-2718.

28. Perales-Calvo J, Lezamiz A, Garcia-Manyes S: The Mechanochemistry of a Structural Zinc Finger. *The journal of physical chemistry letters* 2015:3335-3340.

29. Kropachev KY, Zharkov DO, Grollman AP: Catalytic mechanism of Escherichia coli endonuclease VIII: roles of the intercalation loop and the zinc finger. *Biochemistry* 2006, 45(39):12039-12049.

30. Vallee BL: Zinc biochemistry in normal and neoplastic growth processes. *Experientia* 1977, 33(5):600-601.

31. Hensley MP, Tierney DL, Crowder MW: Zn(II) binding to *Escherichia coli* 70S ribosomes. *Biochemistry* 2011, 50(46):9937-9939.

32. Campbell EA, Greenwell R, Anthony JR, Wang S, Lim L, Das K, Sofia HJ, Donohue TJ, Darst SA: A conserved structural module regulates transcriptional responses to diverse stress signals in bacteria. *Molecular cell* 2007, 27(5):793-805.

33. Vallee BL, Riordan JF: Dynamics of local conformation and enzyme function. *Ciba*

Foundation symposium 1977(60):197-223.

34. Ortiz de Orue Lucana D, Wedderhoff I, Groves MR: ROS-Mediated Signalling in Bacteria: Zinc-Containing Cys-X-X-Cys Redox Centres and Iron-Based Oxidative Stress. *Journal of signal transduction* 2012, 2012:605905.

35. Nagai K, Nakaseko Y, Nasmyth K, Rhodes D: Zinc-finger motifs expressed in *E. coli* and folded in vitro direct specific binding to DNA. *Nature* 1988, 332(6161):284-286.

36. Hanas JS, Hazuda DJ, Bogenhagen DF, Wu FY, Wu CW: Xenopus transcription factor A requires zinc for binding to the 5 S RNA gene. *The Journal of biological chemistry* 1983, 258(23):14120-14125.

37. Prasad AS: Zinc: mechanisms of host defense. *The Journal of nutrition* 2007, 137(5):1345-1349.

38. Rink L, Kirchner H: Zinc-altered immune function and cytokine production. *The Journal of nutrition* 2000, 130(5S Suppl):1407S-1411S.

39. Sargeant HR, Miller HM, Shaw MA: Inflammatory response of porcine epithelial IPEC J2 cells to enterotoxigenic *E. coli* infection is modulated by zinc supplementation. *Molecular immunology* 2011, 48(15-16):2113-2121.

40. Ibs KH, Rink L: Zinc-altered immune function. *The Journal of nutrition* 2003, 133(5 Suppl 1):1452S-1456S.

41. Fraker PJ, King LE, Laakko T, Vollmer TL: The dynamic link between the integrity of the immune system and zinc status. *The Journal of nutrition* 2000, 130(5S Suppl):1399S-1406S.

42. Djoko KY, Ong CL, Walker MJ, McEwan AG: The Role of Copper and Zinc Toxicity in Innate Immune Defense against Bacterial Pathogens. *The Journal of biological chemistry* 2015, 290(31):18954-18961.

43. Beyersmann D, Haase H: Functions of zinc in signaling, proliferation and differentiation of mammalian cells. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2001, 14(3-4):331-341.

44. Zhang B, Guo Y: Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning piglets. *The British journal of nutrition* 2009, 102(5):687-693.

45. Rohweder J, Runkel N, Fromm M, Schulzke JD, Buhr HJ: Zinc acts as a protective agent on the mucosal barrier in experimental TNBS colitis. *Langenbecks Archiv fur Chirurgie Supplement Kongressband Deutsche Gesellschaft fur Chirurgie Kongress* 1998, 115(Suppl I):223-227.

46. Prasad AS: Zinc: role in immunity, oxidative stress and chronic inflammation. *Current opinion in clinical nutrition and metabolic care* 2009, 12(6):646-652.

47. Bray TM, Bettger WJ: The physiological role of zinc as an antioxidant. *Free radical biology & medicine* 1990, 8(3):281-291.

48. Agren MS, Chvapil M, Franzen L: Enhancement of re-epithelialization with topical zinc oxide in porcine partial-thickness wounds. *The Journal of surgical research* 1991,

50(2):101-105.

49. Vahjen W, Pieper R, Zentek J: Increased dietary zinc oxide changes the bacterial core and enterobacterial composition in the ileum of piglets. *Journal of animal science* 2011, 89(8):2430-2439.

50. Li JW, Wang XP, Wang CY, Zhu YL, Li FC: Effects of dietary electrolyte balance on growth performance, nitrogen metabolism and some blood biochemical parameters of growing rabbits. *Asian-Australasian journal of animal sciences* 2013, 26(12):1726-1731.

51. Hahn JD, Baker DH: Growth and plasma zinc responses of young pigs fed pharmacologic levels of zinc. *Journal of animal science* 1993, 71(11):3020-3024.

52. Roselli M, Finamore A, Garaguso I, Britti MS, Mengheri E: Zinc oxide protects cultured enterocytes from the damage induced by *Escherichia coli. The Journal of nutrition* 2003, 133(12):4077-4082.

53. de Queiroz CA, Fonseca SG, Frota PB, Figueiredo IL, Aragao KS, Magalhaes CE, de Carvalho CB, Lima AA, Ribeiro RA, Guerrant RL *et al*: Zinc treatment ameliorates diarrhea and intestinal inflammation in undernourished rats. *BMC gastroenterology* 2014, 14:136.

54. Sargeant HR, McDowall KJ, Miller HM, Shaw MA: Dietary zinc oxide affects the expression of genes associated with inflammation: Transcriptome analysis in piglets challenged with ETEC K88. *Veterinary immunology and immunopathology* 2010, 137(1-2):120-129.

55. Cario E, Jung S, Harder D'Heureuse J, Schulte C, Sturm A, Wiedenmann B, Goebell H, Dignass AU: Effects of exogenous zinc supplementation on intestinal epithelial repair in vitro. *European journal of clinical investigation* 2000, 30(5):419-428.

56. Osredkar J SN: Copper and Zinc, Biological Role and Significance of Copper/Zinc Imbalance. *J Clinic Toxicol* 2011, S3:001.

57. Bednorz C, Oelgeschlager K, Kinnemann B, Hartmann S, Neumann K, Pieper R, Bethe A, Semmler T, Tedin K, Schierack P *et al*: The broader context of antibiotic resistance: zinc feed supplementation of piglets increases the proportion of multi-resistant *Escherichia coli* in vivo. *International journal of medical microbiology : IJMM* 2013, 303(6-7):396-403.

58. Colin F. Mills MSc P, CChem, FRSC, FRSE: Zinc in Human Biology, vol. pp 365-370; 1989.

59. MacDonald RS: The role of zinc in growth and cell proliferation. *The Journal of nutrition* 2000, 130(5S Suppl):1500S-1508S.

60. Sahin K, Sahin N, Kucuk O, Hayirli A, Prasad AS: Role of dietary zinc in heat-stressed poultry: a review. *Poultry science* 2009, 88(10):2176-2183.

61. Tucker HF, Salmon WD: Parakeratosis or zinc deficiency disease in the pig. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine* 1955, 88(4):613-616.

62. Jamall IS, Ally KM, Yusuf S: Acrodermatitis enteropathica: zinc therapy and possible

identification of a carrier state through multiple hair zinc analyses over three decades. *Biological trace element research* 2006, 114(1-3):93-105.

63. Neldner KH, Hambidge KM: Zinc therapy of acrodermatitis enteropathica. *The New England journal of medicine* 1975, 292(17):879-882.

64. Wintergerst ES, Maggini S, Hornig DH: Contribution of selected vitamins and trace elements to immune function. *Annals of nutrition & metabolism* 2007, 51(4):301-323.

65. Rink L, Gabriel P: Zinc and the immune system. *The Proceedings of the Nutrition Society* 2000, 59(4):541-552.

66. Fernandes G, Nair M, Onoe K, Tanaka T, Floyd R, Good RA: Impairment of cell-mediated immunity functions by dietary zinc deficiency in mice. *Proceedings of the National Academy of Sciences of the United States of America* 1979, 76(1):457-461.

67. Fraker PJ, Haas SM, Luecke RW: Effect of zinc deficiency on the immune response of the young adult A/J mouse. *The Journal of nutrition* 1977, 107(10):1889-1895.

68. Wellinghausen N, Kirchner H, Rink L: The immunobiology of zinc. *Immunology today* 1997, 18(11):519-521.

69. Hennig B, Wang Y, Ramasamy S, McClain CJ: Zinc deficiency alters barrier function of cultured porcine endothelial cells. *The Journal of nutrition* 1992, 122(6):1242-1247.

70. Wapnir RA: Zinc deficiency, malnutrition and the gastrointestinal tract. *The Journal of nutrition* 2000, 130(5S Suppl):1388S-1392S.

71. Siefert H WH: Bloodstream infection and endocarditis. *In: Borriello SP, Murray PR, Funke G (eds) Topley and Wilson's Microbiology and MicrobialInfections Vol 1 10th Ed London: Edward Arnold 2005,509-526.*

72. Gordon DM, Cowling A: The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology* 2003, 149(Pt 12):3575-3586.

73. Kaper JB, Nataro JP, Mobley HL: Pathogenic *Escherichia coli*. *Nature reviews Microbiology* 2004, 2(2):123-140.

74. Romer A, Wieler LH, Schierack P: Analyses of intestinal commensal *Escherichia coli* strains from wild boars suggest adaptation to conventional pig production conditions. *Veterinary microbiology* 2012, 161(1-2):122-129.

75. Katouli M, Lund A, Wallgren P, Kuhn I, Soderlind O, Mollby R: Phenotypic characterization of intestinal *Escherichia coli* of pigs during suckling, postweaning, and fattening periods. *Applied and environmental microbiology* 1995, 61(2):778-783.

76. Korolik VV: [Properties of *E. coli*, Salm. typhimurium and Cl. perfringens strains isolated from soil containing bacterial insecticides based on Bac. thuringiensis]. *Gigiena i sanitariia* 1980(4):77-78.

77. Holden NJ, Wright F, Mackenzie K, Marshall J, Mitchell S, Mahajan A, Wheatley R, Daniell TJ: Prevalence and diversity of *Escherichia coli* isolated from a barley trial supplemented with bulky organic soil amendments: green compost and bovine slurry. *Letters in applied microbiology* 2014, 58(3):205-212.

78. Njage PM, Buys EM: Pathogenic and commensal *Escherichia coli* from irrigation water show potential in transmission of extended spectrum and AmpC beta-lactamases determinants to isolates from lettuce. *Microbial biotechnology* 2015, 8(3):462-473.

79. Akiba M, Senba H, Otagiri H, Prabhasankar VP, Taniyasu S, Yamashita N, Lee K, Yamamoto T, Tsutsui T, Ian Joshua D *et al*: Impact of wastewater from different sources on the prevalence of antimicrobial-resistant *Escherichia coli* in sewage treatment plants in South India. *Ecotoxicology and environmental safety* 2015, 115:203-208.

80. Tenaillon O, Skurnik D, Picard B, Denamur E: The population genetics of commensal *Escherichia coli*. *Nature reviews Microbiology* 2010, 8(3):207-217.

81. Blattner FR, Plunkett G, 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF *et al*: The complete genome sequence of *Escherichia coli* K-12. *Science* 1997, 277(5331):1453-1462.

82. Lukjancenko O, Wassenaar TM, Ussery DW: Comparison of 61 sequenced *Escherichia coli* genomes. *Microbial ecology* 2010, 60(4):708-720.

83. Rasko DA, Rosovitz MJ, Myers GS, Mongodin EF, Fricke WF, Gajer P, Crabtree J, Sebaihia M, Thomson NR, Chaudhuri R *et al*: The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. *Journal of bacteriology* 2008, 190(20):6881-6893.

84. Chapman TA, Wu XY, Barchia I, Bettelheim KA, Driesen S, Trott D, Wilson M, Chin JJ: Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Applied and environmental microbiology* 2006, 72(7):4782-4795.

85. Clermont O, Bonacorsi S, Bingen E: Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Applied and environmental microbiology* 2000, 66(10):4555-4558.

86. Semmler T, Eichhorn I, Bethe A, Bauerfeind R, Pickard D, Kingsley RA, Dougan G, Wieler LH: Genome Sequence of Porcine *Escherichia coli* Strain IMT8073, an Atypical Enteropathogenic *E. coli* Strain Isolated from a Piglet with Diarrhea. *Genome announcements* 2013, 1(4).

87. Nataro JP, Kaper JB: Diarrheagenic *Escherichia coli. Clinical microbiology reviews* 1998, 11(1):142-201.

88. Tanih NF, Sekwadi E, Ndip RN, Bessong PO: Detection of pathogenic *Escherichia coli* and Staphylococcus aureus from cattle and pigs slaughtered in abattoirs in Vhembe District, South Africa. *TheScientificWorldJournal* 2015, 2015:195972.

89. Moulin-Schouleur M, Reperant M, Laurent S, Bree A, Mignon-Grasteau S, Germon P, Rasschaert D, Schouler C: Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. *Journal of clinical microbiology* 2007, 45(10):3366-3376.

90. Ochman H, Selander RK: Standard reference strains of *Escherichia coli* from natural populations. *Journal of bacteriology* 1984, 157(2):690-693.

91. Johnson JR, Russo TA: Extraintestinal pathogenic *Escherichia coli*: "the other bad *E coli*". *The Journal of laboratory and clinical medicine* 2002, 139(3):155-162.

92. Bentley R, Meganathan R: Biosynthesis of vitamin K (menaquinone) in bacteria. *Microbiological reviews* 1982, 46(3):241-280.

93. Haller D, Serrant P, Peruisseau G, Bode C, Hammes WP, Schiffrin E, Blum S: IL-10 producing CD14low monocytes inhibit lymphocyte-dependent activation of intestinal epithelial cells by commensal bacteria. *Microbiology and immunology* 2002, 46(3):195-205.

94. Schierack P, Romer A, Jores J, Kaspar H, Guenther S, Filter M, Eichberg J, Wieler LH: Isolation and characterization of intestinal *Escherichia coli* clones from wild boars in Germany. *Applied and environmental microbiology* 2009, 75(3):695-702.

95. Ochoa-Reparaz J, Mielcarz DW, Begum-Haque S, Kasper LH: Gut, bugs, and brain: role of commensal bacteria in the control of central nervous system disease. *Annals of neurology* 2011, 69(2):240-247.

96. Hudault S, Guignot J, Servin AL: *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection. *Gut* 2001, 49(1):47-55.

97. Leatham MP, Banerjee S, Autieri SM, Mercado-Lubo R, Conway T, Cohen PS: Precolonized human commensal *Escherichia coli* strains serve as a barrier to *E. coli* O157:H7 growth in the streptomycin-treated mouse intestine. *Infection and immunity* 2009, 77(7):2876-2886.

98. Schierack P, Kleta S, Tedin K, Babila JT, Oswald S, Oelschlaeger TA, Hiemann R, Paetzold S, Wieler LH: *E. coli* Nissle 1917 Affects Salmonella adhesion to porcine intestinal epithelial cells. *PloS one* 2011, 6(2):e14712.

99. Schierack P, Rodiger S, Kuhl C, Hiemann R, Roggenbuck D, Li G, Weinreich J, Berger E, Nolan LK, Nicholson B *et al*: Porcine *E. coli*: virulence-associated genes, resistance genes and adhesion and probiotic activity tested by a new screening method. *PloS one* 2013, 8(4):e59242.

100. Katouli M: Population structure of gut *Escherichia coli* and its role in development of extra-intestinal infections. *Iranian journal of microbiology* 2010, 2(2):59-72.

101. Macutkiewicz C, Carlson G, Clark E, Dobrindt U, Roberts I, Warhurst G: Characterisation of *Escherichia coli* strains involved in transcytosis across gut epithelial cells exposed to metabolic and inflammatory stress. *Microbes and infection / Institut Pasteur* 2008, 10(4):424-431.

102. MacFie J: Current status of bacterial translocation as a cause of surgical sepsis. *British medical bulletin* 2004, 71:1-11.

103. Kagambega A, Martikainen O, Siitonen A, Traore AS, Barro N, Haukka K: Prevalence of diarrheagenic *Escherichia coli* virulence genes in the feces of slaughtered cattle, chickens, and pigs in Burkina Faso. *MicrobiologyOpen* 2012, 1(3):276-284.

104. Croxen MA, Finlay BB: Molecular mechanisms of *Escherichia coli* pathogenicity. *Nature reviews Microbiology* 2010, 8(1):26-38.

105. Malik A, Toth I, Nagy B: Colonisation of conventional weaned pigs by enteropathogenic *Escherichia coli* (EPEC) and its hazard potential for human health. *Acta veterinaria*

Hungarica 2012, 60(3):297-307.

106. Borges CA, Beraldo LG, Maluta RP, Cardozo MV, Guth BE, Rigobelo EC, de Avila FA: Shiga toxigenic and atypical enteropathogenic *Escherichia coli* in the feces and carcasses of slaughtered pigs. *Foodborne pathogens and disease* 2012, 9(12):1119-1125.

107. Etcheverria AI, Padola NL: Shiga toxin-producing *Escherichia coli*: factors involved in virulence and cattle colonization. *Virulence* 2013, 4(5):366-372.

108. Vallance BA, Chan C, Robertson ML, Finlay BB: Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: emerging themes in pathogenesis and prevention. *Canadian journal of gastroenterology-Journal canadien de gastroenterologie* 2002, 16(11):771-778.

109. Telli M, Guiral E, Martinez JA, Almela M, Bosch J, Vila J, Soto SM: Prevalence of enterotoxins among *Escherichia coli* isolates causing bacteraemia. *FEMS microbiology letters* 2010, 306(2):117-121.

110. Scaletsky IC, Fabbricotti SH, Carvalho RL, Nunes CR, Maranhao HS, Morais MB, Fagundes-Neto U: Diffusely adherent *Escherichia coli* as a cause of acute diarrhea in young children in Northeast Brazil: a case-control study. *Journal of clinical microbiology* 2002, 40(2):645-648.

111. Laarmann S, Schmidt MA: The *Escherichia coli* AIDA autotransporter adhesin recognizes an integral membrane glycoprotein as receptor. *Microbiology* 2003, 149(Pt 7):1871-1882.

112. Johnson AM, Kaushik RS, Francis DH, Fleckenstein JM, Hardwidge PR: Heat-labile enterotoxin promotes *Escherichia coli* adherence to intestinal epithelial cells. *Journal of bacteriology* 2009, 191(1):178-186.

113. Helie P, Morin M, Jacques M, Fairbrother JM: Experimental infection of newborn pigs with an attaching and effacing *Escherichia coli* O45:K"E65" strain. *Infection and immunity* 1991, 59(3):814-821.

114. Hernandes RT, Elias WP, Vieira MA, Gomes TA: An overview of atypical enteropathogenic *Escherichia coli*. *FEMS microbiology letters* 2009, 297(2):137-149.

115. Nguyen Y, Sperandio V: Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Frontiers in cellular and infection microbiology* 2012, 2:90.

116. Deng W, Li Y, Vallance BA, Finlay BB: Locus of enterocyte effacement from Citrobacter rodentium: sequence analysis and evidence for horizontal transfer among attaching and effacing pathogens. *Infection and immunity* 2001, 69(10):6323-6335.

117. Garmendia J, Frankel G, Crepin VF: Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: translocation, translocation, translocation. *Infection and immunity* 2005, 73(5):2573-2585.

118. Rocha SP, Abe CM, Sperandio V, Bando SY, Elias WP: Atypical enteropathogenic *Escherichia coli* that contains functional locus of enterocyte effacement genes can be attaching-and-effacing negative in cultured epithelial cells. *Infection and immunity* 2011, 79(5):1833-1841.

119. Goffaux F, China B, Janssen L, Pirson V, Mainil J: The locus for enterocyte effacement

(LEE) of enteropathogenic *Escherichia coli* (EPEC) from dogs and cats. *Advances in experimental medicine and biology* 1999, 473:129-136.

120. Pelayo JS, Scaletsky IC, Pedroso MZ, Sperandio V, Giron JA, Frankel G, Trabulsi LR: Virulence properties of atypical EPEC strains. *Journal of medical microbiology* 1999, 48(1):41-49.

121. Trabulsi LR, Keller R, Tardelli Gomes TA: Typical and atypical enteropathogenic *Escherichia coli. Emerging infectious diseases* 2002, 8(5):508-513.

122. Frohlicher E, Krause G, Zweifel C, Beutin L, Stephan R: Characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from pigs and sheep. *BMC microbiology* 2008, 8:144.

123. Hernandes RT, Vieira MA, Carneiro SM, Salvador FA, Gomes TA: Characterization of atypical enteropathogenic *Escherichia coli* strains that express typical localized adherence in HeLa cells in the absence of the bundle-forming pilus. *Journal of clinical microbiology* 2006, 44(11):4214-4217.

124. Jafari A, Aslani MM, Bouzari S: *Escherichia coli*: a brief review of diarrheagenic pathotypes and their role in diarrheal diseases in Iran. *Iranian journal of microbiology* 2012, 4(3):102-117.

125. Wu XY, Chapman T, Trott DJ, Bettelheim K, Do TN, Driesen S, Walker MJ, Chin J: Comparative analysis of virulence genes, genetic diversity, and phylogeny of commensal and enterotoxigenic *Escherichia coli* isolates from weaned pigs. *Applied and environmental microbiology* 2007, 73(1):83-91.

126. Otero V, Rodriguez-Calleja JM, Otero A, Garcia-Lopez ML, Santos JA: Genetic characterization of atypical enteropathogenic *Escherichia coli* isolates from ewes' milk, sheep farm environments, and humans by multilocus sequence typing and pulsed-field gel electrophoresis. *Applied and environmental microbiology* 2013, 79(19):5864-5869.

127. Smith JL, Fratamico PM, Gunther NW: Extraintestinal pathogenic *Escherichia coli*. *Foodborne pathogens and disease* 2007, 4(2):134-163.

128. Russo TA, Johnson JR: Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. *The Journal of infectious diseases* 2000, 181(5):1753-1754.

129. Russo TA, Johnson JR: Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. *Microbes and infection / Institut Pasteur* 2003, 5(5):449-456.

130. Johnson JR, Russo TA: Molecular epidemiology of extraintestinal pathogenic (uropathogenic) *Escherichia coli. International journal of medical microbiology : IJMM* 2005, 295(6-7):383-404.

131. Wold AE, Caugant DA, Lidin-Janson G, de Man P, Svanborg C: Resident colonic *Escherichia coli* strains frequently display uropathogenic characteristics. *The Journal of infectious diseases* 1992, 165(1):46-52.

132. Ewers C, Antao EM, Diehl I, Philipp HC, Wieler LH: Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic

potential. *Applied and environmental microbiology* 2009, 75(1):184-192.

133. Bergeron CR, Prussing C, Boerlin P, Daignault D, Dutil L, Reid-Smith RJ, Zhanel GG, Manges AR: Chicken as reservoir for extraintestinal pathogenic *Escherichia coli* in humans, Canada. *Emerging infectious diseases* 2012, 18(3):415-421.

134. Gerjets I, Traulsen I, Reiners K, Kemper N: Comparison of virulence gene profiles of *Escherichia coli* isolates from sows with coliform mastitis and healthy sows. *Veterinary microbiology* 2011, 152(3-4):361-367.

135. Schierack P, Walk N, Ewers C, Wilking H, Steinruck H, Filter M, Wieler LH: ExPEC-typical virulence-associated genes correlate with successful colonization by intestinal *E. coli* in a small piglet group. *Environmental microbiology* 2008, 10(7):1742-1751.

136. Ewers C, Dematheis F, Singamaneni HD, Nandanwar N, Fruth A, Diehl I, Semmler T, Wieler LH: Correlation between the genomic o454-nlpD region polymorphisms, virulence gene equipment and phylogenetic group of extraintestinal *Escherichia coli* (ExPEC) enables pathotyping irrespective of host, disease and source of isolation. *Gut pathogens* 2014, 6:37.

137. Johnson JR, Murray AC, Gajewski A, Sullivan M, Snippes P, Kuskowski MA, Smith KE: Isolation and molecular characterization of nalidixic acid-resistant extraintestinal pathogenic *Escherichia coli* from retail chicken products. *Antimicrobial agents and chemotherapy* 2003, 47(7):2161-2168.

138. Eide DJ: Zinc transporters and the cellular trafficking of zinc. *Biochimica et biophysica acta* 2006, 1763(7):711-722.

139. Wang D, Hurst TK, Thompson RB, Fierke CA: Genetically encoded ratiometric biosensors to measure intracellular exchangeable zinc in *Escherichia coli*. *Journal of biomedical optics* 2011, 16(8):087011.

140. Takahashi H, Oshima T, Hobman JL, Doherty N, Clayton SR, Iqbal M, Hill PJ, Tobe T, Ogasawara N, Kanaya S *et al*: The dynamic balance of import and export of zinc in *Escherichia coli* suggests a heterogeneous population response to stress. *Journal of the Royal Society, Interface / the Royal Society* 2015, 12(106).

141. Gabbianelli R, Scotti R, Ammendola S, Petrarca P, Nicolini L, Battistoni A: Role of ZnuABC and ZinT in *Escherichia coli* O157:H7 zinc acquisition and interaction with epithelial cells. *BMC microbiology* 2011, 11:36.

142. Patzer SI, Hantke K: The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Molecular microbiology* 1998, 28(6):1199-1210.

143. Yatsunyk LA, Easton JA, Kim LR, Sugarbaker SA, Bennett B, Breece RM, Vorontsov, II, Tierney DL, Crowder MW, Rosenzweig AC: Structure and metal binding properties of ZnuA, a periplasmic zinc transporter from *Escherichia coli*. *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* 2008, 13(2):271-288.

144. Grass G, Wong MD, Rosen BP, Smith RL, Rensing C: ZupT is a Zn(II) uptake system in *Escherichia coli*. *Journal of bacteriology* 2002, 184(3):864-866.

145. Grass G, Franke S, Taudte N, Nies DH, Kucharski LM, Maguire ME, Rensing C: The metal permease ZupT from *Escherichia coli* is a transporter with a broad substrate spectrum. *Journal of bacteriology* 2005, 187(5):1604-1611.

146. Myers SA: Zinc transporters and zinc signaling: new insights into their role in type 2 diabetes. *International journal of endocrinology* 2015, 2015:167503.

147. Grass G, Fan B, Rosen BP, Franke S, Nies DH, Rensing C: ZitB (YbgR), a member of the cation diffusion facilitator family, is an additional zinc transporter in *Escherichia coli. Journal of bacteriology* 2001, 183(15):4664-4667.

148. Rensing C, Mitra B, Rosen BP: The zntA gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. *Proceedings of the National Academy of Sciences of the United States of America* 1997, 94(26):14326-14331.

149. Wang D, Hosteen O, Fierke CA: ZntR-mediated transcription of zntA responds to nanomolar intracellular free zinc. *Journal of inorganic biochemistry* 2012, 111:173-181.

150. Wang D, Fierke CA: The BaeSR regulon is involved in defense against zinc toxicity in *E. coli. Metallomics : integrated biometal science* 2013, 5(4):372-383.

151. Bhutta ZA, Bird SM, Black RE, Brown KH, Gardner JM, Hidayat A, Khatun F, Martorell R, Ninh NX, Penny ME *et al*: Therapeutic effects of oral zinc in acute and persistent diarrhea in children in developing countries: pooled analysis of randomized controlled trials. *The American journal of clinical nutrition* 2000, 72(6):1516-1522.

152. Perez VG, Waguespack AM, Bidner TD, Southern LL, Fakler TM, Ward TL, Steidinger M, Pettigrew JE: Additivity of effects from dietary copper and zinc on growth performance and fecal microbiota of pigs after weaning. *Journal of animal science* 2011, 89(2):414-425.

153. Crane JK, Broome JE, Reddinger RM, Werth BB: Zinc protects against Shiga-toxigenic *Escherichia coli* by acting on host tissues as well as on bacteria. *BMC microbiology* 2014, 14:145.

154. Crane JK, Byrd IW, Boedeker EC: Virulence inhibition by zinc in shiga-toxigenic *Escherichia coli*. *Infection and immunity* 2011, 79(4):1696-1705.

155. Crane JK, Naeher TM, Shulgina I, Zhu C, Boedeker EC: Effect of zinc in enteropathogenic *Escherichia coli* infection. *Infection and immunity* 2007, 75(12):5974-5984.

156. Medeiros P, Bolick DT, Roche JK, Noronha F, Pinheiro C, Kolling GL, Lima A, Guerrant RL: The micronutrient zinc inhibits EAEC strain 042 adherence, biofilm formation, virulence gene expression, and epithelial cytokine responses benefiting the infected host. *Virulence* 2013, 4(7):624-633.

157. Huang SX MM, C. Cegielski A, N. Kirkwood R: Effect of dietary zinc supplementation on *Escherichia coli* septicemia in weaned pigs. *Swine Health Prod* 1998, 7(3):109–111.

158. Mellies JL, Thomas K, Turvey M, Evans NR, Crane J, Boedeker E, Benison GC: Zinc-induced envelope stress diminishes type III secretion in enteropathogenic *Escherichia coli*. *BMC microbiology* 2012, 12:123.

159. Xue Y, Osborn J, Panchal A, Mellies JL: The RpoE Stress Response Pathway Mediates Reduction of the Virulence of Enteropathogenic *Escherichia coli* by Zinc. *Applied and environmental microbiology* 2015, 81(11):3766-3774.

160. Sigdel TK, Cilliers R, Gursahaney PR, Thompson P, Easton JA, Crowder MW: Probing the adaptive response of *Escherichia coli* to extracellular Zn(II). *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2006, 19(5):461-471.

161. Easton JA, Thompson P, Crowder MW: Time-dependent translational response of *E. coli* to excess Zn(II). *Journal of biomolecular techniques : JBT* 2006, 17(5):303-307.

162. Hogberg LD, Heddini A, Cars O: The global need for effective antibiotics: challenges and recent advances. *Trends in pharmacological sciences* 2010, 31(11):509-515.

163. Lin J, Nishino K, Roberts MC, Tolmasky M, Aminov RI, Zhang L: Mechanisms of antibiotic resistance. *Frontiers in microbiology* 2015, 6:34.

164. Giedraitiene A, Vitkauskiene A, Naginiene R, Pavilonis A: Antibiotic resistance mechanisms of clinically important bacteria. *Medicina* 2011, 47(3):137-146.

165. Ammor MS, Florez AB, van Hoek AH, de Los Reyes-Gavilan CG, Aarts HJ, Margolles A, Mayo B: Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. *Journal of molecular microbiology and biotechnology* 2008, 14(1-3):6-15.

166. Schwarz S, Chaslus-Dancla E: Use of antimicrobials in veterinary medicine and mechanisms of resistance. *Veterinary research* 2001, 32(3-4):201-225.

167. Thomas CM, Nielsen KM: Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature reviews Microbiology* 2005, 3(9):711-721.

168. Tenover FC: Mechanisms of antimicrobial resistance in bacteria. *The American journal of medicine* 2006, 119(6 Suppl 1):S3-10; discussion S62-70.

169. Szmolka A, Nagy B: Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. *Frontiers in microbiology* 2013, 4:258.

170. Dzidic C SJ, Kos,B: Antibiotic Resistance Mechanisms in Bacteria:Biochemical and Genetic Aspects. *Food Technol Biotechnol* 2008, 46 (1) 11–21 (2008).

171. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ: Molecular mechanisms of antibiotic resistance. *Nature reviews Microbiology* 2015, 13(1):42-51.

172. Vila J, Marcos A, Marco F, Abdalla S, Vergara Y, Reig R, Gomez-Lus R, Jimenez de Anta T: In vitro antimicrobial production of beta-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferase by and susceptibility of clinical isolates of Acinetobacter baumannii. *Antimicrobial agents and chemotherapy* 1993, 37(1):138-141.

173. Hotta K: Biochemical and genetic mechanisms for bacteria to acquire aminoglycoside antibiotic resistance. *Nihon rinsho Japanese journal of clinical medicine* 1997, 55(5):1231-1237.

174. Kehrenberg C, Schwarz S, Jacobsen L, Hansen LH, Vester B: A new mechanism for

chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. *Molecular microbiology* 2005, 57(4):1064-1073.

175. Kumar S FVM: Molecular mechanisms of bacterial resistance to antimicrobial agents *Microbial pathogens and strategies for combating them: science, technology and education (A Méndez-Vilas, Ed)* 2013.

176. Delcour AH: Outer membrane permeability and antibiotic resistance. *Biochimica et biophysica acta* 2009, 1794(5):808-816.

177. Wright GD: Molecular mechanisms of antibiotic resistance. *Chemical communications* 2011, 47(14):4055-4061.

178. Viveiros M, Dupont M, Rodrigues L, Couto I, Davin-Regli A, Martins M, Pages JM, Amaral L: Antibiotic stress, genetic response and altered permeability of *E. coli. PloS one* 2007, 2(4):e365.

179. Nikaido H: Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and molecular biology reviews : MMBR* 2003, 67(4):593-656.

180. Sato K, Nakae T: Outer membrane permeability of Acinetobacter calcoaceticus and its implication in antibiotic resistance. *The Journal of antimicrobial chemotherapy* 1991, 28(1):35-45.

181. Mayrhofer S, Paulsen P, Smulders FJ, Hilbert F: Antimicrobial resistance in commensal *Escherichia coli* isolated from muscle foods as related to the veterinary use of antimicrobial agents in food-producing animals in Austria. *Microbial drug resistance* 2006, 12(4):278-283.

182. Yazdankhah S, Rudi K, Bernhoft A: Zinc and copper in animal feed - development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. *Microbial ecology in health and disease* 2014, 25.

183. Dobrindt U, Agerer F, Michaelis K, Janka A, Buchrieser C, Samuelson M, Svanborg C, Gottschalk G, Karch H, Hacker J: Analysis of genome plasticity in pathogenic and commensal *Escherichia coli* isolates by use of DNA arrays. *Journal of bacteriology* 2003, 185(6):1831-1840.

184. Liu P, Pieper R, Rieger J, Vahjen W, Davin R, Plendl J, Meyer W, Zentek J: Effect of dietary zinc oxide on morphological characteristics, mucin composition and gene expression in the colon of weaned piglets. *PloS one* 2014, 9(3):e91091.

185. Murugaiyan J, Rockstroh M, Wagner J, Baumann S, Schorsch K, Trump S, Lehmann I, Bergen M, Tomm JM: Benzo[a]pyrene affects Jurkat T cells in the activated state via the antioxidant response element dependent Nrf2 pathway leading to decreased IL-2 secretion and redirecting glutamine metabolism. *Toxicology and applied pharmacology* 2013, 269(3):307-316.

186. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG: Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* 2004, 25(9):1327-1333.

187. Berth M, Moser FM, Kolbe M, Bernhardt J: The state of the art in the analysis of two-dimensional gel electrophoresis images. *Applied microbiology and biotechnology*

2007, 76(6):1223-1243.

188. Wareth G, Melzer F, Weise C, Neubauer H, Roesler U, Murugaiyan J: Proteomics-based identification of immunodominant proteins of Brucellae using sera from infected hosts points towards enhanced pathogen survival during the infection. *Biochemical and biophysical research communications* 2015, 456(1):202-206.

189. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M: Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 2005, 21(18):3674-3676.

190. Willenbrock H, Hallin PF, Wassenaar TM, Ussery DW: Characterization of probiotic *Escherichia coli* isolates with a novel pan-genome microarray. *Genome biology* 2007, 8(12):R267.

191. Hayashi T, Makino K, Ohnishi M, Kurokawa K, Ishii K, Yokoyama K, Han CG, Ohtsubo E, Nakayama K, Murata T *et al*: Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA research : an international journal for rapid publication of reports on genes and genomes* 2001, 8(1):11-22.

192. Wang Y, Huang SH, Wass CA, Stins MF, Kim KS: The gene locus yijP contributes to *Escherichia coli* K1 invasion of brain microvascular endothelial cells. *Infection and immunity* 1999, 67(9):4751-4756.

193. Johnson TJ, Wannemuehler YM, Nolan LK: Evolution of the iss gene in *Escherichia coli. Applied and environmental microbiology* 2008, 74(8):2360-2369.

194. Sherlock O, Schembri MA, Reisner A, Klemm P: Novel roles for the AIDA adhesin from diarrheagenic *Escherichia coli*: cell aggregation and biofilm formation. *Journal of bacteriology* 2004, 186(23):8058-8065.

195. Hammar M, Arnqvist A, Bian Z, Olsen A, Normark S: Expression of two csg operons is required for production of fibronectin- and congo red-binding curli polymers in *Escherichia coli* K-12. *Molecular microbiology* 1995, 18(4):661-670.

196. Maheswari UB, Palvai S, Anuradha PR, Kammili N: Hemagglutination and biofilm formation as virulence markers of uropathogenic *Escherichia coli* in acute urinary tract infections and urolithiasis. *Indian journal of urology : IJU : journal of the Urological Society of India* 2013, 29(4):277-281.

197. Uhlich GA, Gunther NWt, Bayles DO, Mosier DA: The CsgA and Lpp proteins of an *Escherichia coli* O157:H7 strain affect HEp-2 cell invasion, motility, and biofilm formation. *Infection and immunity* 2009, 77(4):1543-1552.

198. Zhao L, Chen X, Xu X, Song G, Liu X: Analysis of the AIDA-I gene sequence and prevalence in *Escherichia coli* isolates from pigs with post-weaning diarrhoea and oedema disease. *Veterinary journal* 2009, 180(1):124-129.

199. Schierack P, Walk N, Reiter K, Weyrauch KD, Wieler LH: Composition of intestinal Enterobacteriaceae populations of healthy domestic pigs. *Microbiology* 2007, 153(Pt 11):3830-3837.

200. Schierack P, Steinruck H, Kleta S, Vahjen W: Virulence factor gene profiles of

Escherichia coli isolates from clinically healthy pigs. *Applied and environmental microbiology* 2006, 72(10):6680-6686.

201. Kim KS: *Escherichia coli* translocation at the blood-brain barrier. *Infection and immunity* 2001, 69(9):5217-5222.

202. Vanotti MB, Millner PD, Hunt PG, Ellison AQ: Removal of pathogen and indicator microorganisms from liquid swine manure in multi-step biological and chemical treatment. *Bioresource technology* 2005, 96(2):209-214.

203. Sandvang D: Novel streptomycin and spectinomycin resistance gene as a gene cassette within a class 1 integron isolated from *Escherichia coli*. *Antimicrobial agents and chemotherapy* 1999, 43(12):3036-3038.

204. Allard JD, Bertrand KP: Sequence of a class E tetracycline resistance gene from *Escherichia coli* and comparison of related tetracycline efflux proteins. *Journal of bacteriology* 1993, 175(14):4554-4560.

205. Hellweger FL: *Escherichia coli* adapts to tetracycline resistance plasmid (pBR322) by mutating endogenous potassium transport: in silico hypothesis testing. *FEMS microbiology ecology* 2013, 83(3):622-631.

206. van Den Bogaard AE, London N, Stobberingh EE: Antimicrobial resistance in pig faecal samples from the Netherlands (five abattoirs) and Sweden. *The Journal of antimicrobial chemotherapy* 2000, 45(5):663-671.

207. Guerra B, Junker E, Schroeter A, Malorny B, Lehmann S, Helmuth R: Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *The Journal of antimicrobial chemotherapy* 2003, 52(3):489-492.

208. Szmolka A, Anjum MF, La Ragione RM, Kaszanyitzky EJ, Nagy B: Microarray based comparative genotyping of gentamicin resistant *Escherichia coli* strains from food animals and humans. *Veterinary microbiology* 2012, 156(1-2):110-118.

209. Roberts MC: Update on acquired tetracycline resistance genes. *FEMS microbiology letters* 2005, 245(2):195-203.

210. Schwarz S, Silley P, Simjee S, Woodford N, van Duijkeren E, Johnson AP, Gaastra W: Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *The Journal of antimicrobial chemotherapy* 2010, 65(4):601-604.

211. Kozak GK, Boerlin P, Janecko N, Reid-Smith RJ, Jardine C: Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. *Applied and environmental microbiology* 2009, 75(3):559-566.

212. Scott HM, Campbell LD, Harvey RB, Bischoff KM, Alali WQ, Barling KS, Anderson RC: Patterns of antimicrobial resistance among commensal *Escherichia coli* isolated from integrated multi-site housing and worker cohorts of humans and swine. *Foodborne pathogens and disease* 2005, 2(1):24-37.

213. Mathew AG, Garner KN, Ebner PD, Saxton AM, Clift RE, Liamthong S: Effects of antibiotic use in sows on resistance of *E. coli* and Salmonella enterica Typhimurium in

their offspring. *Foodborne pathogens and disease* 2005, 2(3):212-220.

214. Pettigrew JE: Reduced use of antibiotic growth promoters in diets fed to weanling pigs: dietary tools, part 1. *Animal biotechnology* 2006, 17(2):207-215.

215. Silver S: Bacterial resistances to toxic metal ions--a review. *Gene* 1996, 179(1):9-19.

216. Liedtke J, Vahjen W: In vitro antibacterial activity of zinc oxide on a broad range of reference strains of intestinal origin. *Veterinary microbiology* 2012, 160(1-2):251-255.

217. Noll M, Petrukhin K, Lutsenko S: Identification of a novel transcription regulator from Proteus mirabilis, PMTR, revealed a possible role of YJAI protein in balancing zinc in *Escherichia coli. The Journal of biological chemistry* 1998, 273(33):21393-21401.

218. Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV: Co-selection of antibiotic and metal resistance. *Trends in microbiology* 2006, 14(4):176-182.

219. Cavaco LM, Hasman H, Aarestrup FM: Zinc resistance of Staphylococcus aureus of animal origin is strongly associated with methicillin resistance. *Veterinary microbiology* 2011, 150(3-4):344-348.

220. Yamamoto K, Ishihama A: Transcriptional response of *Escherichia coli* to external zinc. *Journal of bacteriology* 2005, 187(18):6333-6340.

221. Dineley KE, Votyakova TV, Reynolds IJ: Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. *Journal of neurochemistry* 2003, 85(3):563-570.

222. Akerlund T, Nordstrom K, Bernander R: Analysis of cell size and DNA content in exponentially growing and stationary-phase batch cultures of *Escherichia coli. Journal of bacteriology* 1995, 177(23):6791-6797.

223. Salem W, Leitner DR, Zingl FG, Schratter G, Prassl R, Goessler W, Reidl J, Schild S: Antibacterial activity of silver and zinc nanoparticles against Vibrio cholerae and enterotoxic *Escherichia coli*. *International journal of medical microbiology : IJMM* 2015, 305(1):85-95.

224. Hojberg O, Canibe N, Poulsen HD, Hedemann MS, Jensen BB: Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. *Applied and environmental microbiology* 2005, 71(5):2267-2277.

225. Yamamoto O: Influence of particle size on the antibacterial activity of zinc oxide. *International Journal of Inorganic Materials* 2001, 3 (2001) 643–646.

226. Seif S, Kazempour ZB, Pourmand MR, Shahverdi HR, Amanlou M, Bazl R, Nazari ZE, Shahverdi AR: Preparation of ciprofloxacin-coated zinc oxide nanoparticles and their antibacterial effects against clinical isolates of Staphylococcus aureus and *Escherichia coli*. *Arzneimittel-Forschung* 2011, 61(8):472-476.

227. Lu S, Duffin R, Poland C, Daly P, Murphy F, Drost E, Macnee W, Stone V, Donaldson K: Efficacy of simple short-term in vitro assays for predicting the potential of metal oxide nanoparticles to cause pulmonary inflammation. *Environmental health perspectives* 2009, 117(2):241-247.

228. Lee LJ, Barrett JA, Poole RK: Genome-wide transcriptional response of

chemostat-cultured *Escherichia coli* to zinc. *Journal of bacteriology* 2005, 187(3):1124-1134.

229. Cousins RJ, Blanchard RK, Moore JB, Cui L, Green CL, Liuzzi JP, Cao J, Bobo JA: Regulation of zinc metabolism and genomic outcomes. *The Journal of nutrition* 2003, 133(5 Suppl 1):1521S-1526S.

230. Hensley MP, Gunasekera TS, Easton JA, Sigdel TK, Sugarbaker SA, Klingbeil L, Breece RM, Tierney DL, Crowder MW: Characterization of Zn(II)-responsive ribosomal proteins YkgM and L31 in *E. coli*. *Journal of inorganic biochemistry* 2012, 111:164-172.

231. Gauci VJ, Padula MP, Coorssen JR: Coomassie blue staining for high sensitivity gel-based proteomics. *Journal of proteomics* 2013, 90:96-106.

232. Cain JA, Solis N, Cordwell SJ: Beyond gene expression: the impact of protein post-translational modifications in bacteria. *Journal of proteomics* 2014, 97:265-286.

233. Xie Y, He Y, Irwin PL, Jin T, Shi X: Antibacterial activity and mechanism of action of zinc oxide nanoparticles against Campylobacter jejuni. *Applied and environmental microbiology* 2011, 77(7):2325-2331.

234. Jung IL, Kim IG: Transcription of ahpC, katG, and katE genes in *Escherichia coli* is regulated by polyamines: polyamine-deficient mutant sensitive to H2O2-induced oxidative damage. *Biochemical and biophysical research communications* 2003, 301(4):915-922.

235. Mulvey MR, Switala J, Borys A, Loewen PC: Regulation of transcription of katE and katF in *Escherichia coli*. *Journal of bacteriology* 1990, 172(12):6713-6720.

236. Skorko-Glonek J, Zurawa D, Kuczwara E, Wozniak M, Wypych Z, Lipinska B: The *Escherichia coli* heat shock protease HtrA participates in defense against oxidative stress. *Molecular & general genetics : MGG* 1999, 262(2):342-350.

237. Singh D, Chang SJ, Lin PH, Averina OV, Kaberdin VR, Lin-Chao S: Regulation of ribonuclease E activity by the L4 ribosomal protein of *Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America* 2009, 106(3):864-869.

238. Seaver LC, Imlay JA: Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *Journal of bacteriology* 2001, 183(24):7173-7181.

239. Visick JE, Clarke S: RpoS- and OxyR-independent induction of HPI catalase at stationary phase in *Escherichia coli* and identification of rpoS mutations in common laboratory strains. *Journal of bacteriology* 1997, 179(13):4158-4163.

240. Nandakumar R, Espirito Santo C, Madayiputhiya N, Grass G: Quantitative proteomic profiling of the *Escherichia coli* response to metallic copper surfaces. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2011, 24(3):429-444.

241. Fayet O, Ziegelhoffer T, Georgopoulos C: The groES and groEL heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *Journal of bacteriology* 1989, 171(3):1379-1385.

242. Zhou Y, Xu YB, Xu JX, Zhang XH, Xu SH, Du QP: Combined toxic effects of heavy metals and antibiotics on a Pseudomonas fluorescens strain ZY2 isolated from swine wastewater. *International journal of molecular sciences* 2015, 16(2):2839-2850.

243. Holzel CS, Muller C, Harms KS, Mikolajewski S, Schafer S, Schwaiger K, Bauer J: Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environmental research* 2012, 113:21-27.

244. Spain A, Alm E: Implications of Microbial Heavy Metal Tolerance in the Environment. *Reviews in Undergraduate Research* 2003, Vol. 2, 1-6 , 2003.

245. Randall LP, Woodward MJ: The multiple antibiotic resistance (mar) locus and its significance. *Research in veterinary science* 2002, 72(2):87-93.

246. Nishino K, Nikaido E, Yamaguchi A: Regulation and physiological function of multidrug efflux pumps in *Escherichia coli* and *Salmonella*. *Biochimica et biophysica acta* 2009, 1794(5):834-843.

247. Mahendran KR, Kreir M, Weingart H, Fertig N, Winterhalter M: Permeation of antibiotics through *Escherichia coli* OmpF and OmpC porins: screening for influx on a single-molecule level. *Journal of biomolecular screening* 2010, 15(3):302-307.

248. Otto K, Hermansson M: Inactivation of ompX causes increased interactions of type 1 fimbriated *Escherichia coli* with abiotic surfaces. *Journal of bacteriology* 2004, 186(1):226-234.

249. Nikaido H: Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Seminars in cell & developmental biology* 2001, 12(3):215-223.

250. Liu X, Ferenci T: Regulation of porin-mediated outer membrane permeability by nutrient limitation in *Escherichia coli. Journal of bacteriology* 1998, 180(15):3917-3922.

251. Castillo-Keller M, Vuong P, Misra R: Novel mechanism of *Escherichia coli* porin regulation. *Journal of bacteriology* 2006, 188(2):576-586.

252. Charlson ES, Werner JN, Misra R: Differential effects of yfgL mutation on *Escherichia coli* outer membrane proteins and lipopolysaccharide. *Journal of bacteriology* 2006, 188(20):7186-7194.

253. Mogk A, Deuerling E, Vorderwulbecke S, Vierling E, Bukau B: Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Molecular microbiology* 2003, 50(2):585-595.

254. Egler M, Grosse C, Grass G, Nies DH: Role of the extracytoplasmic function protein family sigma factor RpoE in metal resistance of *Escherichia coli*. *Journal of bacteriology* 2005, 187(7):2297-2307.

255. Sayed A, Matsuyama S, Inoue K, Alsina J, Cai F, Chen J, Inouye M: ATPase and GTPase activities copurifying with GTP-binding proteins in *E. coli. Journal of molecular microbiology and biotechnology* 2000, 2(3):261-263.

256. Freestone P, Trinei M, Clarke SC, Nystrom T, Norris V: Tyrosine phosphorylation in *Escherichia coli. Journal of molecular biology* 1998, 279(5):1045-1051.

257. Farris M, Grant A, Richardson TB, O'Connor CD: BipA: a tyrosine-phosphorylated

GTPase that mediates interactions between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells. *Molecular microbiology* 1998, 28(2):265-279.

258. Qi SY, Li Y, Szyroki A, Giles IG, Moir A, O'Connor CD: Salmonella typhimurium responses to a bactericidal protein from human neutrophils. *Molecular microbiology* 1995, 17(3):523-531.

259. Grant AJ, Farris M, Alefounder P, Williams PH, Woodward MJ, O'Connor CD: Co-ordination of pathogenicity island expression by the BipA GTPase in enteropathogenic *Escherichia coli* (EPEC). *Molecular microbiology* 2003, 48(2):507-521.

260. Rowe S, Hodson N, Griffiths G, Roberts IS: Regulation of the *Escherichia coli* K5 capsule gene cluster: evidence for the roles of H-NS, BipA, and integration host factor in regulation of group 2 capsule gene clusters in pathogenic *E. coli. Journal of bacteriology* 2000, 182(10):2741-2745.

261. Vimr ER, Troy FA: Identification of an inducible catabolic system for sialic acids (nan) in *Escherichia coli. Journal of bacteriology* 1985, 164(2):845-853.

262. Vogel-Scheel J, Alpert C, Engst W, Loh G, Blaut M: Requirement of purine and pyrimidine synthesis for colonization of the mouse intestine by *Escherichia coli. Applied and environmental microbiology* 2010, 76(15):5181-5187.

263. Moreau PL: The lysine decarboxylase CadA protects *Escherichia coli* starved of phosphate against fermentation acids. *Journal of bacteriology* 2007, 189(6):2249-2261.

264. Maurelli AT, Fernandez RE, Bloch CA, Rode CK, Fasano A: "Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of Shigella spp. and enteroinvasive *Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America* 1998, 95(7):3943-3948.

265. Vazquez-Juarez RC, Kuriakose JA, Rasko DA, Ritchie JM, Kendall MM, Slater TM, Sinha M, Luxon BA, Popov VL, Waldor MK *et al*: CadA negatively regulates *Escherichia coli* O157:H7 adherence and intestinal colonization. *Infection and immunity* 2008, 76(11):5072-5081.

266. Torres AG, Vazquez-Juarez RC, Tutt CB, Garcia-Gallegos JG: Pathoadaptive mutation that mediates adherence of shiga toxin-producing *Escherichia coli* O111. *Infection and immunity* 2005, 73(8):4766-4776.

267. Prigent-Combaret C, Vidal O, Dorel C, Lejeune P: Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli. Journal of bacteriology* 1999, 181(19):5993-6002.

268. Wang S, Deng K, Zaremba S, Deng X, Lin C, Wang Q, Tortorello ML, Zhang W: Transcriptomic response of *Escherichia coli* O157:H7 to oxidative stress. *Applied and environmental microbiology* 2009, 75(19):6110-6123.

SUPPLEMENTAL INFORMATION

Supplemental Table 1: 253 Virulence associated genes (VAGs) screened in IMT29408 and IMT8073.

Gene	IMT29408	IMT8073	Gene	IMT29408	IMT8073	Gene	IMT29408	IMT8073
aafA	-	-	*cdtA_III*	-	-	*escC*	-	+
aah	-	-	*cdtA_IV*	-	-	*escD*	-	+
aap	-	-	*cdtB*	-	-	*escF*	-	+
aatA	-	-	*cdtB_III*	-	-	*escJ*	-	+
ada	+	+	*cdtB_IV*	-	-	*escN*	-	+
adhesin v1	-	+	*cdtC*	-	-	*escR*	-	+
adhesin v2	-	-	*cdtC_III*	-	-	*escS*	-	+
afaA	-	-	*cdtC_IV*	-	-	*escT*	-	+
afaB	-	-	*cfa*	+	+	*escU*	-	+
afaC	-	-	*chuA*	-	-	*escV*	-	+
afaD	-	-	*cif*	-	-	*espA*	-	+
afaE	-	-	*cnf1*	-	-	*espB*	-	-
afaE-3	-	-	*cnf2*	-	-	*espC*	-	-
afaE-7	-	-	*csgA*	+	+	*espD*	-	+
afaE-8	-	-	*cvaB*	-	-	*espF*	-	-
afaF	-	-	*cvaC*	-	-	*espG*	-	+
aggA	-	-	*cvi*	-	-	*espH*	-	+
aggB	-	-	*daaA-E*	-	-	*espK*	-	+
aggC	-	-	*daaE*	-	-	*espL2*	-	-
aggD	-	-	*daaF*	-	-	*espP*	-	-
aggR	-	-	*dra2E*	-	-	*est1*	-	-
aidA	+	+	*draA*	-	-	*est2*	-	-
aslA	-	-	*draB*	-	-	*etsA*	-	+
astA/East-1	-	+	*draC*	-	-	*etsB*	-	-
bfpA	-	-	*draD*	-	-	*etsC*	-	-
bfpB	-	-	*draE*	-	-	*f1c focA*	-	-
bfpC	-	-	*draP*	-	-	*faeG*	-	-
bfpD	-	-	*eae*	-	+	*fedA*	-	-
bfpE	-	-	*eae_theta*	-	+	*fedE*	-	-
bfpF	-	-	*ecpR*	+	+	*fedF*	-	-
bfpG	-	-	*efa1/lifA*	-	-	*fimC*	+	+
bfpH	-	-	*ehxA*	-	+	*fimF*	+	+
bfpI	-	-	*ehxB*	-	+	*fimG*	+	+
bfpJ	-	-	*ehxC*	-	+	*fimH*	+	+
bfpK	-	-	*ehxD*	-	+	*Flaggelin FliC*	-	-
bfpL	-	-	*eitA*	-	−	*focA*	+	+
bfpM	-	-	*eitB*	-	−	*focC*	-	-
bfpP	-	-	*eitC*	-	−	*focG*	-	-
bfpU	-	-	*eltA*	-	−	*focH*	-	-
bmaE	-	-	*eltB*	-	-	*fyuA*	-	-
cdtA	-	-	*enterotoxin*	-	-	*gafD*	-	-

"-": negative; "+": positive. IMT29408: *E.coli* commensal strain; IMT8073: pathogenic *E.coli* (aEPEC).

Gene	IMT29408	IMT8073	Gene	IMT29408	IMT8073	Gene	IMT29408	IMT8073
genetic_island_GimB_1	-	-	*nleE*	-	+	*stx2A*	-	-
genetic_island_GimB_2	-	-	*nleF*	-	+	*stx2B*	-	-
genetic_island_GimB_3	-	-	*nleH1*	-	+	*subA*	-	-
genetic_island_GimB_4	-	-	*nleH2*	-	+	*subB*	-	-
genetic_island_GimB_5	-	-	*ompA*	+	+	*tccP_espFu*	-	-
genetic_island_GimB_6	-	-	*ompT*	+	+	*tia*	-	-
hek/hra	-	-	*paa*	-	+	*tir*	-	-
hlyA_v1	-	+	*papA*	-	-	*toxB*	-	-
hlyA_v2	-	-	*papC*	-	-	*traJ*	-	-
hlyC	-	-	*papE*	-	-	*traT*	-	+
hlyD	-	-	*papF*	-	-	*tsh*	-	-
hlyF	-	-	*papG*	-	-	*upaG*	-	-
ibeA	-	-	*papG_II*	-	-	*ureA*	-	-
ibeB	+	+	*papG_III*	-	-	*ureB*	-	-
ibeC	+	+	*pet*	-	-	*ureC*	-	-
icsA	-	-	*pic*	-	-	*ureD*	-	-
iha	-	-	*pks*	-	-	*ureE*	-	-
ipaA	-	-	*puvA*	-	-	*ureF*	-	-
ipaB	-	-	*rtx*	-	-	*ureG*	-	-
ipaC	-	-	*saa*	-	-	*usp*	-	-
ipaD	-	-	*sat*	-	-	*vat*	-	-
ipaH	-	-	*sepA*	-	-	*virA*	-	-
ipgD	-	-	*sepD*	-	+	*virG*	-	-
ireA	-	-	*sepL*	-	+	*ybtA*	-	-
iroE	-	-	*sepQ*	-	+	*ybtU*	-	-
iroN	-	-	*sepZ*	-	-	*yqi*	-	-
iroN v2	-	-	*set1A*	-	-			
irp1	-	-	*sfaA*	-	-			
irp2	-	-	*sfaB*	-	-			
iss	+	+	*sfaC*	-	-			
iucC	-	-	*sfaD*	-	-			
iucD	-	-	*sfaE*	-	-			
iutA	-	-	*sfaF*	-	-			
iutA v2	-	-	*sfaG*	-	-			
kpsD	-	-	*sfaH*	-	-			
kpsE	-	-	*sfaS*	-	-			
kpsF	-	-	*ShET1*	-	-			
kpsM	-	-	*ShET2*	-	-			
kpsMT_II	-	-	*sigA*	-	-			
kpsT	-	-	*sinH*	-	-			
kpsX	-	-	*sitA_episomal*	-	-			
lifaA	-	-	*sitB_episomal*	-	-			
malX	+	+	*sitC_episomal*	-	-			
map	-	+	*sitD_episomal*	-	-			
matB	+	+	*slyA*	+	+			
neuC	-	-	*sta*	-	-			
nfaE	-	-	*sta1*	-	-			
nleA	-	+	*sta3*	-	-			
nleB1	-	+	*stb*	-	-			
nleB2	-	+	*stcE*	-	+			
nleC	-	-	*stx1A*	-	-			
nleD	-	-	*stx1B*	-	-			

"-": negative; "+": positive. IMT29408: *E.coli* commensal strain; IMT8073: pathogenic *E.coli* (aEPEC).

Supplemental table 2: Antibiotic resistance genes screened

Gene name	IMT29408	IMT8073	Gene name	IMT29408	IMT8073
aac6Ib	-	-	*strBshort*	-	+
aac6Ibcr	-	-	*sul1gene*	-	-
aadA1	-	-	*sul1short*	-	-
aadA12	-	-	*sul2gene*	-	+
aadA13	-	-	*sul2short*	-	+
aadA14	-	-	*sul3gene*	-	-
aadA16	-	-	bla_{TEM1}	-	+
aadA2	-	-	bla_{TEM2}	-	-
aadA5	+	-	*tetAgene*	+	-
aadA6	-	-	*tetAPCR*	+	-
aadA7	-	-	*tetAshort*	+	-
aadA9	-	-	*tetBgene*	-	-
blaTEM	-	-	*tetBPCR*	-	-
ctxm7475	-	-	*tetBshort*	-	-
ctxmI	-	-	*tetCgene*	-	-
ctxmII	-	-	*tetCPCR*	-	-
ctxmIIIV	-	-	*tetCshort*	-	-
ctxmIV	-	-			
oqxA	-	-			
oqxB	-	-			
OXAgr1	-	-			
OXAgr10	-	-			
OXAgr2	-	-			
qepA	-	-			
qnrA	-	-			
qnrBI	-	-			
qnrBII	-	-			
qnrBIII	-	-			
qnrBIV	-	-			
qnrC	-	-			
qnrD	-	-			
qnrS	-	-			
qnrVC136	-	-			
qnrVC45	-	-			
SHV	-	-			
strAgene	-	+			
strAPCR	-	+			
strAshort	-	+			
strBPCR	-	+			

"-": negative; "+": positive. IMT29408: *E.coli* commensal strain; IMT8073: pathogenic *E.coli* (aEPEC).

Supplemental table 3: The detailed information of differentially expressed proteins identified in *E. coli* IMT29408 (A) and IMT8073 (B) under exposure to 1 mM zinc chloride

A: Differentially expressed proteins identified in *E. coli* IMT29408 under exposure to 1 mM zinc chloride

Gene name	NCBI Acc. No.	Uniprotkb No.	Mass	Score	Peptides matches	p*I*	Protein coverage %
2 h zinc vs 2 h control							
degP	gi|15799845	P0C0V1	49438	134	2	8.65	7%
fabF	gi|386618670	G0D6R3	41963	134	9	5.4	36%
manX	gi|15831781	P69799	35026	316	16	5.74	55%
mdh	gi|2289309	P61889	30051	121	9	5.38	52%
pgk	gi|486272698	N3RQY8	41260	397	20	5.08	61%
	gi|15803460	Q8XD03	41276	280	3	5.08	11%
rbsB	gi|485843904	I5EZK5	30433	176	6	6.85	27%
rpoA	gi|487470900	M8R3K3	36673	141	8	5.03	27%
tsf	gi|487424998	M8N6X2	30476	95	6	5.22	26%
yhdH	gi|15803786	Q8X9C1	34887	117	10	5.63	34%
5 h zinc vs 5 h control							
degP	gi|15799845	P0C0V1	49438	134	2	8.65	7%
fabF	gi|386618670	G0D6R3	41963	134	9	5.4	36%
rplD	gi|15803846	P60725	22101	94	6	9.72	37%
tnaA	gi|386706983	H9UYR7	49934	147	9	5.31	22%
tpiA	gi|486375362	L4HCP1	27098	88*	3	5.64	23%
	gi|486185246	L3D2R1	27296	89*	6	5.64	35%
tsx	gi|15829718	P0A928	33568	169	4	5.07	24%
ychF	gi|446428007	D7XUT1	39998	92	5	4.87	20%
ahpC	gi|486400833	N3Y9T7	20818	83*	9	5.13	57%
pflB	gi|487636021	N1SN28	85534	139	22	5.65	30%
rffG	gi|386619664	G0D388	40848	91	7	5.4	37%
2 h zinc vs 5 h zinc only							
argS	gi|585333150		58336	90*	8	5.2	16%
asnS	gi|487417619	M8MI31	52768	98	13	5.17	33%
fabI	gi|559173289	V6P9J3	26119	96	11	7.71	41%
groES	gi|15804734	P0A6G1	10381	106	5	5.15	47%
mdh	gi|2289309	P61889	30051	121	9	5.38	52%
talB	gi|209399547	B3BSW7	37690	150	10	5.29	40%
accA	gi|15799867	P0ABD6	35333	82*	2	5.76	8%
adhE	gi|446223768	I2ZWA1	96561	121	9	6.27	15%
fabF	gi|386618670	G0D6R3	41963	171	9	5.4	36%
fumB	gi|545293352	T9N0N8	60557	168	10	5.92	25%
fusA	gi|385251657	E4PBL6	77444	306	16	5.2	31%
	gi|15803853	P0A6N0	77704	79*	3	5.24	6%
	gi|15803853	P0A6N0	77704	112	4	5.24	9%
guaB	gi|486392364	L4J3L4	52247	149	13	6.02	43%
hyaB	gi|16128939	P0ACD8	66895	89*	11	5.61	19%
ompC	gi|6650193	Q9RH85	40474	121	3	4.55	11%
ompX	gi|15800566	P0A919	18648	109	5	6.56	33%
pykA	gi|15802267	Q8XCJ4	51562	139	2	6.67	7%
rplJ	gi|485795535	H4R9B9	15390	280	8	7.85	56%
serS	gi|485734300	N3W3M1	48645	93	12	5.29	23%

surA	gi|446723141	E8J134	47235	101	9	6.34	17%
tufA	gi|15803852	P0A6N3	43427	69*	1	5.3	4%
	gi|485700076	D8AII6	44525	247	19	5.2	52%
	gi|485700076	D8AII6	44525	144	8	5.2	25%

Shared proteins identified between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control in IMT29408

Gene name	NCBI Acc. No.	Uniprotkb No.	Protein Name	Mass	Score	Peptides matches	pI	Protein coverage %
aspA	gi|510926982	S1J2V9	Aspartate ammonia-lyase	51653	146	10	5.3	27%
dps	gi|440182	P0ABT2	DNA protection during starvation protein	18698	76*	2	5.72	15%
gadB	gi|15801645	P69911	Glutamate decarboxylase	53204	174	3	5.29	11%
glpK	gi|446058939	I2U0M3	Glycerol kinase	56422	142	14	5.43	29%
guaA	gi|545247705	T8CZP6	GMP synthase [glutamine-hydrolyzing]	59031	96	13	5.19	31%
manX	gi|545165604	T6B7W0	PTS system mannose-specific EIIAB component	35042	329	17	5.74	60%
pckA	gi|487372189	N4FQP5	Phosphoenolpyruvate carboxykinase	59870	250	10	5.46	31%
pfkA	gi|485974648	N3GN38	6-phosphofructokinase	31398	104	14	5.36	43%
pflB	gi|487636021	N1SN28	Formate acetyltransferase	85534	139	22	5.65	30%
pgk	gi|486272698	N3RQY8	Phosphoglycerate kinase	41260	397	20	5.08	61%
rpoA	gi|487470900	M8R3K3	DNA-directed RNA polymerase, alpha subunit	36673	141	8	5.03	27%
sodB	gi|5902908	Q9R3B9	Iron-containing superoxide dismutase	16240	284	3	5.65	28%
tpx	gi|15801846	P0A864	Thiol peroxidase	17995	128	2	4.75	20%
treC	gi|485719985	L4HAY4	trehalose-6-phosphate hydrolase	64146	261	24	5.49	37%
udp	gi|486435677	N4AIZ0	Uridine phosphorylase	27262	156	4	5.71	13%
yhdH	gi|15803786	Q8X9C1	Dehydrogenase	34887	117	10	5.63	34%
ackA	gi|1359437	P0A6A3	Acetate kinase	43488	165	3	5.76	14%
deoB	gi|15804955	P0A6K8	Phosphopentomutase	44684	143	11	5.11	28%
fabZ	gi|571241951	W1CZ44	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	12557	111	3	6.84	39%
pgi	gi|487506036	M8T4H7	Glucose-6-phosphate isomerase	61601	96	13	5.85	39%
pgk	gi|519085274	–	Phosphoglycerate kinase	35548	89*	7	4.81	26%
rffG	gi|386619664	G0D388	dTDP-glucose 4,6-dehydratase	40848	91*	7	5.4	37%
rplD	gi|446346541	I2Z5T4	50S ribosomal protein L4	22101	94	6	9.72	37%
rpsA	gi|559165069	V6Q0S0	30S ribosomal protein S1	60710	212	10	4.98	26%
tig	gi|147989	P0A850	Trigger factor	47994	302	3	4.73	13%
tpiA	gi|486375362	L4HCP1	Triosephosphate isomerase	27098	88*	3	5.64	23%

B: Differentially expressed proteins identified in *E. coli* IMT8073 under exposure to 1 mM zinc chloride

Gene name	NCBI Acc. No.	Uniprotkb No.	Mass	Score	Peptides matches	pI	Protein coverage %
2 h zinc vs 2 h control							
clpB	gi|476115185	M8RBG7	89278	110	17	5.37	31%
katE	gi|486421966	A0A029PN67	84273	100	15	5.47	23%
5 h zinc vs 5 h control							
accC	gi|545283515	T9DSM8	49749	97	8	6.48	21%
asnS	gi|486026580	N3ITJ4	52809	80*	11	5.28	40%
aspA	gi|485708063	E6B483	52966	198	13	5.14	40%
atpA	gi|146323	P0ABB0	55476	122	12	5.93	34%
atpD	gi|446112654	H4I3E2	50367	284	17	4.9	58%
	gi|487380991	N3ZWI4	50309	135	13	4.9	41%
	gi|446112655	A0A024Z7C1	50350	106	11	4.97	39%
degP	gi|408103926	K3B9B3	45242	102	5	8.35	23%
fabF	gi|445966664	D8C603	43263	132	13	5.51	54%
hybC	gi|742941075	Q0TDB6	62881	100	11	5.77	32%

pyrG	gi|427227085	L0Z8F3	60023	137	13	5.7	38%
tnaA	gi|54401938	Q5UEV2	53874	103	15	6	29%
	gi|486184856	L3D397	53167	121	12	5.88	29%
tsf	gi|485733330	N2Y652	30476	146	13	5.22	48%
cadA	gi|486147025	L2VLC1	81634	133	15	5.91	25%
minD	gi|724482146	A0A0A6RV77	15697	76*	5	4.78	49%
nanA	gi|291426834	F3VAS9	31958	83*	10	5.45	46%
nusA	gi|485893092	N3FDK3	55003	100	19	4.54	44%
pflB	gi|487413284	M8LIQ2	85607	95	21	5.69	32%
udp	gi|545169159	T6GIC0	27471	154	9	5.98	66%
2 h zinc vs 5 h zinc only							
aspA	gi|545252726	T8HLR0	52940	95	8	5.19	23%
	gi|749046403	UPI000589D9D7	24254	91	7	5.31	35%
	gi|485708063	E6B483	52966	198	13	5.14	40%
	gi|345368514	G2BH83	29725	90*	8	6.13	33%
glnA	gi|693117502	A0A071FSQ2	52157	226	15	5.21	53%
glpQ	gi|446701763	F4UP12	40915	142	16	5.44	57%
	gi|446701763	F4UP12	40915	90*	10	5.44	41%
groS	gi|446949021	C2DT52	10409	82*	4	5.15	34%
hybC	gi|742941075	Q0TDB6	62881	100	11	5.77	32%
lysS	gi|447217834	A0A071G9V6	57861	279	28	5.11	55%
pdxJ	gi|486272280	_	26554	80*	6	5.61	48%
prfB	gi|498414361	C9QZM5	41339	145	14	4.64	39%
adhE	gi|446223768	I2ZWA1	96561	102	11	6.27	20%
cadA	gi|486147025	L2VLC1	81634	133	15	5.91	25%
eutD	gi|693095044	A0A069X9C2	77338	190	22	5.28	36%
fabF	gi|445966664	D8C603	43263	93	12	5.51	49%
fbaA	gi|485696065	D8A3F5	39337	114	5	5.52	23%
	gi|485696065	D8A3F5	39337	145	9	5.52	35%
fumA	gi|754640265	_	51103	78*	7	5.99	20%
	gi|693244587	_	60581	109	10	5.88	26%
glmS	gi|446256193	D8CBD2	66907	100	17	5.65	42%
glnS	gi|486155807	L2WVT4	63981	105	10	5.82	25%
glyS	gi|486109353	L1GHS8	73676	182	23	5.36	44%
gnd	gi|489671025	N3KFX6	51529	141	10	5.06	34%
hemX	gi|446061230	B7NTE2	42922	108	12	4.61	37%
nusA	gi|485893092	N3FDK3	55003	100	19	4.54	44%
oppA	gi|330911113	_	62717	82*	11	5.95	32%
pflB	gi|487158424	N4C999	85519	91	15	5.63	22%
	gi|487413284	M8LIQ2	85607	99	14	5.69	23%
pnp	gi|535305294	T7YBB6	75420	166	20	5.16	32%
	gi|487368093	N4EQ89	77041	152	14	5.08	24%
pspE	gi|446024904	V6FQD2	39912	90*	9	6.01	30%
pta	gi|693095044	A0A069X9C2	77338	283	22	5.28	37%
	gi|446008868	G0FE24	77397	177	17	5.23	32%
pyk	gi|345355713	G1ZJ87	48979	139	7	5.65	27%
	gi|487401705	N4TJB6	51035	104	5	5.77	18%
rho	gi|383476813	I0VX80	43066	115	7	7.12	20%
rplI	gi|486164894	L2Z6C8	15772	99	7	6.17	54%
rpoA	gi|571187252	W1G5Y9	33988	96	9	5.03	33%

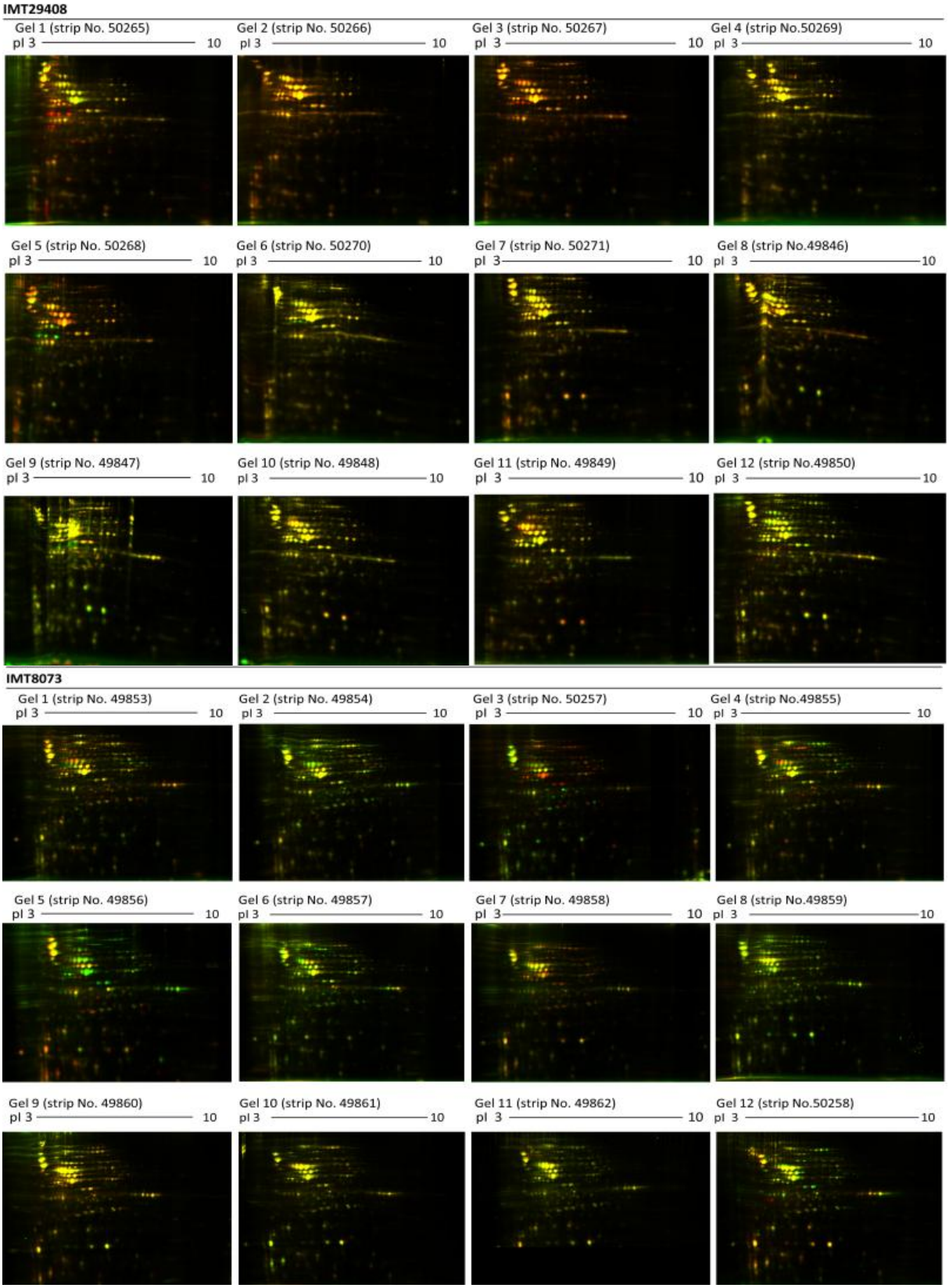
tdcE	gi|447215560	K3F7M9	85611	171	24	5.69	34%
	gi|447215560	K3F7M9	85611	290	21	5.69	32%
	gi|447215560	K3F7M9	85611	86*	15	5.69	27%
tuf	gi|537056256	U1BAJ2	27465	135	4	4.79	21%
	gi|91074389	Q1R5U4	45023	129	18	5.25	56%
	gi|571181793	W1F549	46141	82*	9	5.51	33%
	gi|485710622	E7I2X2	41091	134	17	5.06	62%
	gi|300453428	D8AII6	44525	97	7	5.2	27%
typA	gi|764144094	–	31769	78*	7	6.45	26%
ychF	gi|486403401	L4KJU5	39969	85*	9	4.95	43%

Shared proteins identified between 2 h zinc vs. 5 h zinc and 2 h control vs. 5 h control in IMT8073

Gene name	NCBI Acc.No.	Uniprotkb No.	Protein Name	Mass	Score	Peptides matches	pI	Protein coverage %
accA	gi|485963370	N3HBM7	Acetyl-CoA carboxylase, carboxyl transferase	35264	193	17	5.6	61%
deoC	gi|390913638	I5Z5J8	Deoxyribose-phosphate aldolase	26431	377	7	5.22	44%
dps	gi|486207822	N3Q5G0	DNA protection during starvation protein	18680	103	10	5.72	63%
ftnA	gi|485790492	H4MAH8	Ferritin	17885	137	8	4.77	64%
ftsZ	gi|408133010	K3DH22	Cell division protein	39052	95	9	4.74	21%
gadA	gi|499389176	G7R2D9	Glutamate decarboxylase	55737	98	8	5.3	23%
guaA	gi|545245697	T7XMX0	GMP synthase partial	56216	81*	9	5.36	25%
pckA	gi|487372189	N4FQP5	Phosphoenolpyruvate carboxykinase	59870	167	11	5.46	33%
pyrG	gi|427227085	L0Z8F3	CTP synthase	60023	137	13	5.7	38%
sodB	gi|485952415	N4QK72	Superoxide dismutase	21284	107	8	5.58	68%
ackA	gi|477395042	N4KJ72	Acetate kinase	42766	113	14	5.76	53%
atpA	gi|486198537	L3I3T9	ATP synthase subunit alpha	55446	128	14	5.8	35%
atpD	gi|446112655	A0A024Z7C1	ATP synthase subunit beta	50350	106	11	4.97	39%
citF	gi|545288138	T9KPC3	Citrate lyase alpha chain	55616	99	12	5.91	30%
cysK	gi|486279515	L3UPT8	Cysteine synthase A	34539	89*	8	5.83	51%
degP	gi|408103926	K3B9B3	Serine endoprotease	45242	102	5	8.35	23%
fusA	gi|693078987	A0A073Q6D3	Elongation factor G	77646	129	15	5.27	31%
grpE	gi|486231436	L3P3D5	Co-chaperone GrpE	21844	100	3	4.67	26%
grxB	gi|476214227	M8YTQ2	Glutaredoxin, GrxB family	22268	99	11	5.94	59%
guaB	gi|545251032	T8FMD3	Inosine-5'-monophosphate dehydrogenase	52403	106	11	6.13	41%
ompW	gi|476303042	M9FKG8	Outer membrane protein W	19971	136	7	6.03	46%

"Dark grey": up-regulated proteins; "light grey": down-regulated proteins; "-" representing unknown Uniprotkb number. NCBI Acc. No. is the accession number of the protein identified by comparing the peptide sequence with NCBI database. The uniprotkb No. is the accession number used to cite Uniprotkb entries. Mass (molecular weight) calculated from the identified protein sequence. pI (isoelectronic point) calculated from the identified protein sequence. Some proteins, such as, Pgk and TpiA, were identified in more than one analyzed spot. The MOWSE scores of some proteins did not meet statistically significant difference (student's t test, $p>0.05$), marked as "*", while the number of matched peptides was > 2 and these proteins were ranked top in the list of protein hits, therefore, we chose and considered these proteins as successful identification in order to obtain more information about the effects of zinc on *E. coli* at proteomic level. IMT29408: *E.coli* commensal strain; IMT8073: pathogenic *E.coli* (aEPEC).

Supplemental Figure 1: Overlaid DIGE images across all the gels in IMT29408 and IMT8073.



Overlaid DIGE images of Cy3+Cy5 and detailed information were accorded with Table 2-5 and 2-6.

PUBLICATIONS AND CONFERENCES

A：From the present work (doctoral thesis)

a. Publications

1. **Yun Lu***, Astrid Bethe*, Christoph Weise, Carmen Bednorz, Torsten Semmler, Uwe Roesler, Jürgen Zentek, Lothar H. Wieler, Jayaseelan Murugaiyan. Effects of zinc on intracellular zinc accumulation and proteomic profiles in wild-type commensal *Escherichia coli* isolated from piglets. (* joint first authors) (In preparation)

2. **Yun Lu**, Astrid Bethe, Lothar H.Wieler. The mechanistic impacts of high dietary zinc supplementation on intestinal functionality, *Escherichia coli* and antibiotic resistance (review). (In preparation).

b. Oral presentations

1. "Doctoral Symposium DRS Biomedical Sciences-PHD-symposium". Title: Preliminary proteomics analysis of porcine pathogenic *Escherichia coli* induced by zinc exposure.

03/2015, Berlin, Germany

2. "8th PHD-symposium-bringing future scientists together & DRS presentation seminar". Title: The effects of zinc on protein expression encoded by virulence genes of diarrheagenic *Escherichia coli*. ISBN9783863873264. 07/2013, Berlin, Germany

c. Poster

1. "Microbiology and Infection 2014 - 4th Joint Conference of the German Society for Hygiene and Microbiology (DGHM) and the Association for General and Applied Microbiology (VAAM)". Title: Preliminary proteomic analysis of porcine commensal *Escherichia coli* revealed clues to its response to zinc. Abstract band: FTP18. ISSN09470867.

10/2014, Dresden, Germany

d. Conference attendances

1. "National Symposium on Zoonosis Research" 10/2014, Berlin, Germany

2. "Summer School of Intergrated Research Training Group (Biology of Nutrition)"

07/2014, Berlin, Germany

3. “International Workshop on “Nutrition and Intestinal Microbiota-Host Interaction in pig”
10/2013, Berlin, Germany

4. Workshop of the SFB852 “Nutrition and intestinal microbiota - host interactions in pig”
11/2011, Berlin, Germany

B: From the previous work

1. **Yun Lu***, Ailing Liu*, Xiangmei Zhou*, Mohammed Kouadir, Wei Zhao, Siming Zhang, Xiaomin Yin, Lifengyang, and Deming Zhao (2012). Prion peptide PrP106-126 induces iNOS and pro-inflammatory cytokine gene expressions through the activation of NF-κB in Ana-1 macrophages. *DNA and Cell Biology*. 31(5): 833-838. (* joint first authors)

2. Wei Zhao*, Xiangmei Zhou*, **Yun Lu**, Yun Peng, Zhu Lin. Jingjun Lin, Lifeng Yang, Xiaomin Yin, and Deming Zhao (2014). Mycobacterium Bovis Ornithine Carbamoyltransferase, MB1684, induces Proinflammatory Cytokine Gene Expression by Activating NF-κB in Macrophages. *DNA and Cell Biology.* 33(5): 311-319. (* joint first authors)

3. Mohammed Kouadir*, Lifeng Yang*, Rongrong Tan, Fushan Shi, **Yun Lu,** Siming Zhang, Xiaomin Yin, Xiangmei Zhou, and Deming Zhao (2012). CD36 participates in PrP106-126 induced activation of BV2 microglial cells. *PLOSONE*. 7(1):e30756. (* joint first authors)

4. Siming Zhang*, Lifeng Yang*, Mohammed Kouadir, Rongrong Tan, **Yun Lu**, Jiaxin Chang, Binrui Xu, Xiaomin Yin, Xiangmei Zhou, and Deming Zhao (2013). PP2 and piceatannol inhibitor PrP106-126 induced iNOS activation mediated by CD36 in BV2 microglia. Acta Biochimica et Biophysica Sinica. 45: 763–772. (* joint first authors)

5. **Yun Lu**, Deming Zhao, Xiaomin Yin (2011). The determination of natural attenuation curve of infectious bronchitis virus (IBV). *Chinese Journal of Veterinary Medicine.* 47(3):3-6.

6. **Yun Lu**, Deming Zhao, Lifeng Yang (2011). The study of biological characteristics of scrapie 263K strain injected hamsters. *China Animal Husbandry &Veterinary Medicine*. 38(3): 112-115.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, 04.09.2015 Yun Lu