ANTIBIOTICS RESISTANCE AND REGULATORY MECHANISM OF VIRULENCE EXPRESSION IN *SALMONELLA*

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Antibiotics Resistance and Regulatory Mechanism of Virulence Expression in *Salmonella*

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CERTIFICATE OF ORIGINALITY

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Abstract

Bacterial infections have become an important public health issue worldwide. Combating bacterial infections has been commonplace in human history. Among various bacterial species, *Salmonella enterica* is one of the most important human pathogens. In recent years, acquisition of antibiotics resistance genes by clinically important *Salmonella* strains and invasive infection of high virulence *Salmonella* has become a global concern.

Co-existence of the Transferable Mechanisms of Quinolone Resistance (TMQR) genes *oqxAB* and *aac(6')-Ib-cr* in *Salmonella* is often associated with expression of fluoroquinolone resistance in this important pathogen. However, *Salmonella* that carried the plasmid-borne genes (Tn*6010-oqxABR*) may not express fluoroquinolone-resistance phenotype. The works described in this thesis aimed to investigate the molecular mechanisms underlying regulation of fluoroquinolone susceptibility in *Salmonella* by the *oqxAB* genes, and elucidate the global regulatory mechanisms involved in control of expression of plasmid-borne genes.

In addition to antibiotics resistance, the works in this thesis also described different virulence-encoding mechanisms in *Salmonella*. It should be noted that *Salmonella* is a notorious foodborne pathogen which comprises strains that exhibit varied ability to cause human infection. In previous reports, over-expression of various virulence determination factors was observable in the high virulence *Salmonella* strains. However, the molecular mechanisms underlying expression of virulence in *Salmonella* is still poorly defined. This work therefore aimed at elucidating the regulatory networks that underlie expression of virulence phenotypes in *Salmonella*. In particular, the

relationship between the level of double-stranded RNA (dsRNA) and phenotypic virulence in *Salmonella* was discovered in this study. The molecular mechanism concerned is described and discussed in detail in this thesis. Identification of these virulence regulatory network in *Salmonella* shall facilitate development of novel antimicrobial strategies based on suppression of virulence expression and survival fitness, which potentially offer a solution to the global public health problems caused by this important pathogen.

Publications Produced During the Course of This Study

1. Ni, H. †, **Chan, K. W. B.** †, Cheng, Q., Chen, K., Xie, M., Wang, H., ... & Chen, S.* (2022). A novel clinical therapy to combat infections caused by Hypervirulent Carbapenem-Resistant Klebsiella pneumoniae. *Journal of Infection*, *85*(2), 174-211.
2. **Chan, B. K. W.**, Wong, M. H. Y., Chan, E. W. C., & Chen, S.* (2022). Transcriptional Regulation and Functional Characterization of the Plasmid-Borne oqxAB Genes in Salmonella Typhimurium. *Microbiology Spectrum*, *10*(2), e02170-21.
3. **Chan, K. W.** †, Liu, C. Y. †, Wong, H. Y., Chan, W. C., Wong, K. Y., & Chen, S.* (2022). Specific Amino Acid Substitutions in OXA-51-Type β-Lactamase Enhance Catalytic Activity to a Level Comparable to Carbapenemase OXA-23 and OXA-24/40. *International journal of molecular sciences*, *23*(9), 4496.
4. Po, K. H. L. †, Chow, H. Y. †, Cheng, Q., **Chan, B. K. W.**, Deng, X., Wang, S., ... & Chen, S.* (2021). Daptomycin exerts bactericidal effect through induction of excessive ROS production and blocking the function of stress response protein Usp2. *Natural Sciences*, *1*(2), e10023.
5. Rui Zhang†, Chris Y. Cheung†, Sang-Uk Seo†, Hang Liu, Lakhansing Pardeshi, Koon Ho Wong, Larry M. C. Chow, Mary P. Chau, Yixiang Wang, Ah Ra Lee, Woon Yong Kwon, Sheng Chen, **Bill Kwan-wai Chan**, Kenneth Wong, Richard K. W. Choy and Ben C. B. Ko* (2021). RUVBL1/2 Complex Regulates Pro-Inflammatory Responses in Macrophages via Regulating Histone H3K4 Trimethylation. *Frontiers in immunology*, *12*, 679184.
6. Wong, M. H. Y. †, **Chan, B. K. W.** †, Chan, E. W. C., & Chen, S.* (2019). Over-Expression of IS Aba1-Linked Intrinsic and Exogenously Acquired OXA Type Carbapenem-Hydrolyzing-Class D-ß-Lactamase-Encoding Genes Is Key Mechanism Underlying Carbapenem Resistance in Acinetobacter baumannii. *Frontiers in microbiology*, *10*, 2809.

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Brief Overview of this Thesis

The work presents in this thesis describes the transcriptional regulation and function of the plasmid-borne *oqxAB* gene, including its role in contributing to fluoroquinolone resistance as well as its effect in expression of the virulence phenotype in *Salmonella spp.*. Furthermore, the molecular network of virulence regulation in *Salmonella* was elucidated. The discovery of a novel pathway that controls the virulence level of *Salmonella* is being discussed in this thesis.

Chapter I provides a brief background knowledge of *Salmonlla* species including the basic microbiologic characteristics and epidemiological features of this pathogen. The brief literature review also provides current knowledge in antibiotic resistance and virulence expression in *Salmonlla spp.*. Different antibiotics resistance and virulence factors reported from other studies are summarised.

Chapter II describes the transcriptional regulation mechanisms and functional characteristics of the *oqxAB* genes in *Salmonella*. In this study, we discovered that the expression level of *oqxR* in strains carrying the plasmid-borne *oqxABR* cluster was lower than its chromosomal counterpart due to truncation of specific sequences, and that only an optimal level of OqxAB could act synergistically with the aac(6')-Ib-cr gene product to confer fluoroquinolone resistance in *Salmonella*. Global transcriptional regulators in *Salmonella* were also found to play a role in regulating expression of the plasmid-borne *oqxAB* genes. Most of the materials described in this chapter have been published in *Microbiology Spectrum*.

Chapter III describes the finding that the *oqxABR* locus plays a role in reducing the virulence level of *Salmonlla* by inducing iNOS expression in macrophage to enhance the ability to adapt to survive in the host. The results of the study indicate that expression of iNOS in macrophage cells was upregulated when *oqxABR* was present in *Salmonella*, resulting in reduction in the virulence level of *Salmonella* in RAW264.7 infection model and mouse infection model; however, the potential of gastrointestinal tract colonisation was not affected. We hypothesize that the *oqxABR* operon may render *S*. Typhimurium more adaptive to its host by reducing the virulence level, thereby enhancing survival fitness of host and facilitating inter-host transmission.

Chapter IV describes the novel virulence regulatory networks in *Salmonella* Enteritidis. In this chapter, the molecular mechanisms underlying expression of virulence in *Salmonella* were investigated by genomic, transcriptomic and phenotypic analyses. Our data show that the genes responsible for maltose transport, citrate metabolism, VitB12 biosynthesis, propanediol utilization and hydrogen production are important for expression of the virulence phenotype in *Salmonella*. The regulators *tdcA, yaiV, yncC, yhjB, ramA, stpA* and *rnc* were found to play a role in regulating expression of virulence genes in *Salmonella* based on analysis of gene deletion mutants. The data show that *yhjB* and *yaiV* play a role similar to that of a known virulence regulator *hilD*. Importantly, deletion of *yhjB* or *yhjB* alone resulted in decreased invasiveness, reduced survival inside macrophages, reduced invasion to different organs, and lower mortality in animal experiments. Most of the materials in this chapter is under review in *npj biofilm and microbiome*.

Chapter V describes the role of ribonuclease III in *Salmonella* in enhancing survival against the host immune responses. Transcriptome analysis shows that RNase III

ribonuclease was highly expressed in the high virulence strains. This high expression level of RNase III ribonuclease was found to enhance expression of the superoxide dismutase SodA, which is an essential determinant of survival fitness of *Salmonella* under the oxidative stress elicited by the host immune system. On the other hand, it was found that the double stranded RNA (dsRNA) released from *Salmonella* could trigger immune response of the host; hence high-level expression of RNase III ribonuclease could help reduce the amount of dsRNA accumulated in the bacterial cell and minimize the host immune response. These findings facilitate development of novel antimicrobial treatments through suppression of virulence expression and survival fitness of this important pathogen.

Chapter VI summarizes the findings reported in this thesis. One concludion is that the combined effect of expression of gene *aac(6')-Ib-cr* and a specific level of expression of *oqxAB* may confer fluoroquinolone resistance in *Salmonella*. The regulatory functions of the *ramA* and *oqxR* genes were elucidated. We found that the virulence level of *Salmonella* could be reduced by expression of the *oqxABR* locus. This Chapter also summarizes the mechanism of *Salmonlla* pathogenesis and the roles of a wide range of genes that encode regulatory and metabolic functions and mediate expression of virulence phenotypes in *Salmonella*. The two important global regulatory genes *yhjB* and *yaiV* were found to regulate multiple secondary regulator genes of virulence expression in *S*. Enteritidis. In addition, the finding that dsRNA plays an important role in regulating the expression of Mn-SOD and induction of the host immune response is discussed. Importantly, over-expression of RNase III in *Salmonella* plays a key role in suppressing this induction process, resulting in a milder immune response inducible by *Salmonella*. These novel findings pave the way to devise effective approaches to attenuate bacterial virulence by suppressing expression of specific housekeeping and

stress response genes, as well as their ability to degrade dsRNA, a key factor that triggers the host immune response.

CHAPTER I-Introduction

Microbiology of Salmonella

Salmonella enterica is an ubiquitous enteric bacterium which was first identified in the year 1886 by Daniel E. Salmon[27]. *Salmonellae* are Gram-negative, facultative aerobic, generally motile, rod shaped bacteria[125]. They can grow in common laboratory conditions such as Luria-Bertani or others nutrient media. All *Salmonella enterica* strains are non-lactose fermenter. This phenomenon is probably due to the fact that the lac operon is truncated in the *Salmonella* genome. It was reported that the *lac* operon of *Salmonella spp.* contains the intact *lacI*, *lacY* and *lacZ* gene, and an incomplete *lacA* gene[75]. Most of the *Salmonellae* are aerogenic except for the serovar *Typhi*[125]. Typically, *Salmonella spp.* are hydrogen sulphide-producing. Thus, it can be isolated by the XLT4 agar where it grows to form black centred colonies as a result of the reaction between iron salt and hydrogen sulphide[24]. *Salmonella enterica* is divided into six subspecies; currently over 2500 different serovars were identified by the White-Kauffmann-Le Minor scheme. More than half of the serovars belonged to the subspecies *S. enterica*. These serovars are generally divided into two types, Typhoidal and non-typhoidal serotypes[48]. The non-typhoidal serovars represented the ubiquitous serovars among different serotypes of *S. enterica*, the main host of which are widely distributed among both cold blood and warm blood animals[27]. On the other hand, the host of typhoidal serovars are restricted to human[43].

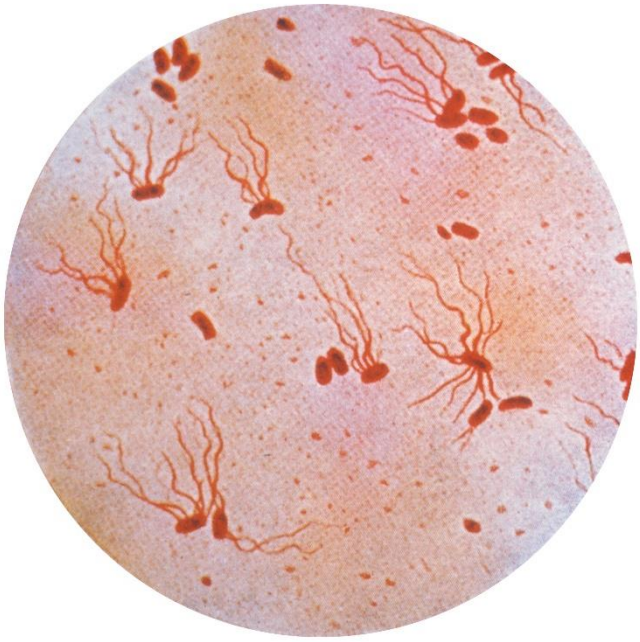

Figure 1.1 The microscopic view of *Salmonella* Typhi using Gram-stain technique. (Picture by US CDC, 1954)

Epidemiology of Salmonella infections

The diseases caused by *Salmonella enterica* had already been documented for centuries; at present, *Salmonella* cause over 1 million cases of foodborne illness in the United States each year [1]. Infections of *Salmonellae* are usually due to consumption of contaminated food or water. The typhoidal *Salmonellae* such as *S. Typhi* are obligate pathogens of human beings which cause life-threatening typhoid or paratyphoid fever[43]. In contrast to non-typhoidal *Salmonellae*, it is invasive and causes systemic disease, in which the organisms concerned can be found in both the intestinal tract and the bloodstream of the patients[26]. Typhoid fever is usually endemic in developing countries. In the late 19th century, it was estimated that 16 million illnesses and 600,000 deaths were due to typhoid fever annually[25]. The latest figure provided by the World Health Organization (WHO) showed that an estimated 11 to 20 million cases occurred each year, resulting in 128,000 to 161,000 deaths. The reduced incidence was due to

invention of vaccine against typhoid fever.

In contrast to the few typhoidal serovars, non-typhoidal serovars comprise a large number of serotypes. The non-typhoidal *Salmonella* diseases are typically transmitted to human through contaminated food or water. Contrary to typhoidal infection, infections due to non-typhoidal serovars are usually not invasive. In most cases, non-typhoidal *Salmonellae* only cause self-limiting diarrhoea in healthy individuals. Rare cases of invasive bloodstream and viscera infections mostly occur in high risk individuals such as immunocompromised patients[40]. Although infection of non-typhoidal *Salmonellae* are generally milder, it is much more common worldwide and is not limited to developing countries[43]. According to the statistics, *Salmonella* was the second most common causative agent of food-borne infections in the United States. The 5 most common serotypes were Enteritidis, Typhimurium, Newport, Javiana, and the monophasic variant of Typhimurium, respectively[86]. In recent years, antibiotic resistant non-typhoidal *Salmonellae* have become a global public health concern. *Salmonella enterica* consists of several types of efflux systems that pump antibiotics out of the cell. For example, the AcrAB-TolC is an effective efflux pump which confers a wide spectrum of antibiotic resistance and is expressed under the induction by indole, a metabolite produced by *Escherichia coli*[94]. This phenomenon suggests that gene regulation of *S. enterica* is highly dependent on the extracellular environment, especially the chemical signals originated from other species of intestinal microbes. In a study in the year 2011, it was shown that the gene expression level of *Salmonella* Pathogenicity Island (SPI) was up-regulated during colonization in the mucosa of intestine of new-born chicken[33], providing high adaptability of *Salmonella* during the process of colonizing the gastrointestinal tract of various animals and rendering *Salmonellae* a highly successful pathogen of both animals and human.

Overview of antibiotic resistance in Salmonella

Non-typhoidal *Salmonellae* are among the principal bacterial pathogens implicated in food-borne gastroenteritis worldwide[46]. The increasing prevalence of multidrug resistant *Salmonellae* has undermined the usefulness of older antimicrobial agents, hence fluoroquinolones and the extended spectrum cephalosporins have become the drugs of choice for treatment of acute gastroenteritis caused by *Salmonellae* and other enteric pathogens. Plasmid mediated quinolone resistance (PMQR) genes such as *qnrA, qnrB, qnrC, qnrD, qnrS, qepA* and *aac(6')Ib-cr* have been recovered in bacterial pathogens, conferring quinolone resistance in these organisms. Recently, a novel transmissible Resistance-Nodulation- Division (RND) efflux pump, OqxAB, has increasingly been reported in different bacterial pathogens. OqxAB was able to mediate expression of resistance towards chloramphenicol and nalidixic acid, and cause reduction in susceptibility towards ciprofloxacin (MIC between 0.06~0.25µg/ml)[51]. More recently, *oqxAB* was reported to be prevalent in organisms isolated from pork as well as pig farms in China [16, 134]. It has been shown *oqxAB* was increasingly detected among clinical isolates of *S.* Typhimurium from 2006 onwards[130]. In addition, our data also suggested a strong linkage between the presence of the *oqxAB* gene and the development of resistance to the fluoroquinolones and other antimicrobial compounds. Among *oqxAB* positive *S.* Typhimurium, about 100%, 94% and 56% of the isolates were resistant to nalidixic acid, ciprofloxacin, and the ACSSuT agents respectively, yet the rate of resistance to these antimicrobials in *oqxAB*-negative S. Typhimurium were only 48%, 11% and 13% respectively. The rapid dissemination of *oqxAB* in *S.* Typhimurium recovered from clinical setting suggests that this element may also contribute to virulence expression as well as defence against host stress response, presumably in the same manner as that of its chromosomal counterpart *acrAB*[133]. As an exogenous efflux pump component, the exact regulatory mechanism

of *oqxAB* as well as its function in *Salmonella* remains to be elucidated and is further discussed in this thesis.

Overview of Salmonella virulence

Based on the results of preliminary studies, the virulence phenotype of *Salmonella* was regarded as being not strictly defined by the genetic trait of specific strain but the expression level of specific housekeeping and stress response genes. These genes include the *araC* family transcriptional regulators *hilD* and *hilC*, invasion protein regulator *hilA*, the two-component system *phoQ*, carbon storage regulator *csrA*, and the RNase III. Our aim is to investigate the mechanisms of the regulation of virulence gene expression and identify the global regulators of virulence expression. Once the global regulator is being targeted, it will be possible to shut down expression of virulence genes in bacteria. This strategy represents an alternative way for controlling bacterial infections apart from using traditional methods such like antibiotics. This virulence suppression strategy will also facilitate development of new approaches for treatment of infections caused by antibiotic-resistant bacteria.

Pathogenic bacterial infection has been a perplexing problem since before the historical record was available. Human had developed multiple approaches to combat bacterial infections for centuries. The current approaches to control infectious disease mainly focus on development of potent antibiotics and attenuation of virulence. However, rapid evolution of bacteria generates different novel resistant mechanisms and highly pathogenic strains which compromise the current efforts of infection control. The evolution of bacteria was found to be associated with multiple factors including dissemination of drug resistant strains among natural hosts, horizontal gene transfer of resistance-encoding genetic elements, antibiotics usage which impose pressure for selection of resistant organisms, and economic activities that promote spread of

resistant organisms[42, 63, 76]. These factors continue to impose selection pressure on drug resistant organisms and the global pool of such strains has expanded to a size that it is impossible to revert the situation. Thus, a novel approach is necessary to control drug resistant microbial infections and safeguard human health.

Salmonella enterica is an important food-borne pathogen in most industrialized countries. Thus, the virulence phenotype of *Salmonellae* had been investigated by many studies and surveillances. In most cases, the non-typhoidal serovars such as Typhimurium and Enteritidis cause self-limited gastroenteritis, intestinal inflammation and diarrhoea. However, it was reported that up to 5% of non-typhoidal serovars infection cases in developed countries result in invasive bacteraemia and focal systemic infections[116]. It was discovered that some uncommon non-typhoidal serotypes such as *S.* Choleraesuis and *S.* Dublin exhibit higher chance of causing severe infection when compared to other non-typhoidal serotypes[65]. This phenomenon infers that the genetic environment of different serotypes may confer different degrees of virulence. In addition, a reported high virulence *S.* Typhimurium ST313 strain isolated from Malawi and Kenya in Africa exhibited genome degradation[71]. This further confirmed that the genetic environment plays a role in determining the virulence level of *Salmonellae*.

To launch infection, the bacterial cells must proliferate and synthesize a complex array of gene products which are essential for invasion and survival in the host environment[1, 9, 36, 68]. Previous studies have identified the common genetic differences which distinguished pathogenic *salmonellae* from their non-pathogenic relatives, the identified genes in so-called *Salmonella* pathogenicity islands (SPI)[68]. The *Salmonella* pathogenicity island 1 (SPI-1) is required for efficient invasion of the intestinal epithelium, and *Salmonella* pathogenicity island 2 (SPI-2) is required for the

bacterial proliferation and survival within macrophages and the progression of systemic infections[9]. The different virulence levels that existed between unrelated strains was believed to be due to the genetic changes that arise in response to environmental stress.

Virulence expression of *Salmonella* involved expression of a series of gene products, including different proteins and peptides. In a preliminary study, we found that the virulence level of *Salmonella* was determined by the intrinsic mechanisms of regulation of virulence genes expression. We were unable to find any suspected genomic differences among the *Salmonella* strains that exhibit dramatic variation in virulence phenotypes through typical genetic analysis. Thus, the mechanisms of regulating the expression of virulence genes may involve an alternative approach. Transcription of virulence genes is subjected to regulation by a complex molecular network involving different regulatory proteins and effector molecules. It is important to identify the key factors involved in virulence regulation in *Salmonella*.

Metabolic Adaptation

As a member of *Enterobacteriaceae*, the habitats of *Salmonella* are widely distributed in the environment. Apart from water and soil, they can be found in various kinds of animal hosts. The natural hosts of *Salmonella* include mammals, birds, reptiles, amphibians and insects[57]. *Salmonella* infections are mainly initiated through ingestion of *Salmonella* contaminated food or drinks. At the beginning of the infection process, *Salmonella* must be able to survive and proliferate in the GI tract of human. The environment of GI tract has many unique characteristics not present in the natural environment. Some may be beneficial for survival of micro-organisms, but some are detrimental. As the physiological function of GI-tract involves digestion of food, a wide variety of nutrients will be generated, such as simple form or polymerized form of saccharides, amino acids, peptides and proteins, lipids, and many others essential

ions[36]. However, the types of nutrients available are highly dependent on the diet of the host and highly variable. On the other hand, the GI-tract is colonized by various microbiota that compete for different nutrients. Therefore, availability of nutrients in the GI-tract is unstable and shortage of specific nutrients is a common event. The metabolism of *Salmonella* involves highly complex catabolic and biosynthetic pathways that confer the ability to utilize different organic chemicals as their energy sources and synthesize different essential cellular components, rendering *Salmonella* the flexibility and robustness to survive in complex and hostile environment. This is because the highly adaptable metabolic pathways allow *Salmonella* to select the best nutrient sources to maximize their survival fitness[28].

Flagella

To effectively obtain the required nutrients, the redundant metabolic pathway offers a passive way to achieve this goal. However, *Salmonella* also developed a cellular component known as flagella for cell propulsion and actively migrate to the target environmental niches to obtain the essential nutrients. The flagella filaments not only propel the bacterial cells in a forward motion or enable the cell to orientate randomly, but also act in response to different chemical stimuli [32]. Under certain stimulatory conditions, the flagella can also secrete virulence related effector proteins such as SptP and SopE, in addition to their primary functions[66]. The basic structure of flagella consists of a few components. The major part which is exposed on the cells surface are proteins *fliD* (the cap of flagella), *fliC* (the filament part), and *flgE* (the hook). Beneath the cell surface, there is the basal segment of flagella which consists of the outer membrane anchored *flgH* (L-ring anchor protein), a periplasmic rod, *flgI* (a periplasmic P-ring), *fliF* (an inner membrane MS-ring), *motA* and *motB* (stator element proteins of motor), *fliM* and *fliN* (C-ring protein), and various flagella protein export apparatus

including *flhA, flhB*, *fliO*, *fliP*, *fliQ*, and *fliR* [23]. As a motility organelle, it contains a motor part which generates the kinetic energy it needs. Flagellum is powered by proton motive force and undergo a rotary motion in both clockwise or anticlockwise directions to drive the cell moving forward or backward [66]. This proton motive force must be generated from the energy storage molecule ATP, in which the energy is released upon lysis by the enzyme ATPase encoded by *fliI* which forms a complex with *fliH* and *fliJ*. (**Figure 1.2**).

The function of flagella in *Salmonella* have been found to be important for adherence and invasion to epithelial cells *in vitro*. An invasion potential analysis by using differentiated Caco-2 cells showed that flagella was not necessary for invasion by *Salmonella* serotype Enteritidis, but can speed up the invasion process [120].

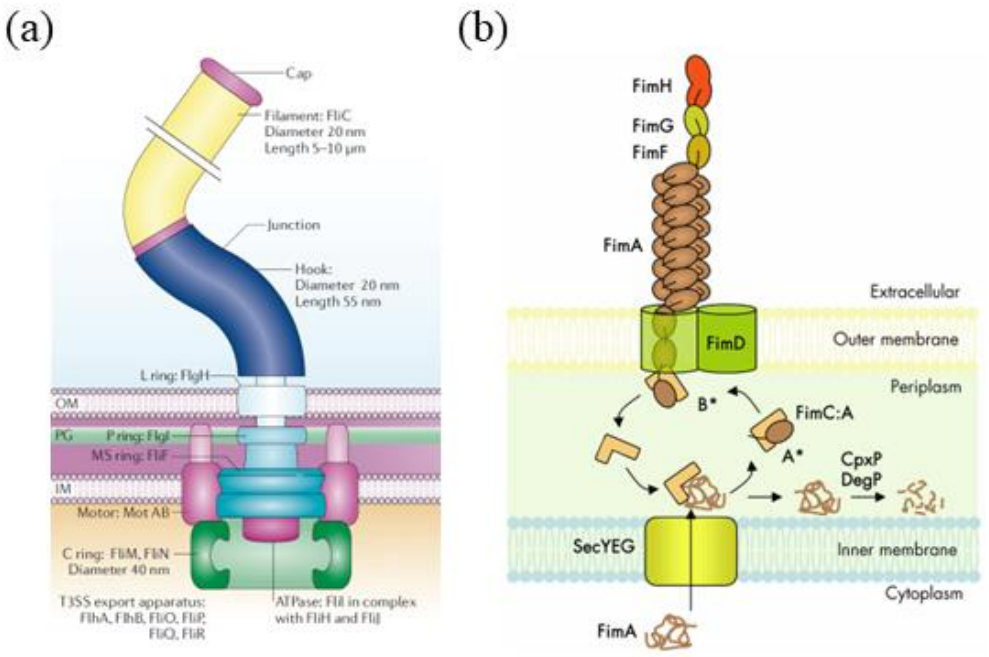

Figure 1.2. (a)Structure of the flagella. OM, outer membrane of bacteria; **PG, peptidoglycan; IM, inner membrane. [23]. (b)Structure of the fimbriae[37].**

Fimbriae

Apart from being able to adapt utilization of a wide range of chemicals to survive in the

GI tract, *Salmonella* may invade into the epithelial cells and cause systemic infections by effectively attaching to one or more cell types of intestinal tissue. This process often involves several types of fimbriae, which is a hair-like structure composed of proteins polymers and distributed on the bacterial cell surface, and is believed to play a critical role in virulence expression by allowing bacteria to interact with host cells and other solid substrates. In fact, not all fimbriae perform specific functions required for infection. Fimbriae operons of various types are widely distributed in the bacterial genome, among them type-I fimbriae can be found in many Gram-negative bacteria, providing general adhesive functions [34]. On the other hand, some fimbriae, such as those encoded by plasmid-borne genes, are found in *Salmonella* only, such fimbriae may provide more specific functions involved in virulence expression and infections. Such functions include specific binding to microfold cells (M cells) in the lymphoid tissue of small intestine.

To date, a total of 13 fimbriae loci have been identified, 4 of which are well defined in *S.* Enteritidis. Those 4 loci belong to type-I fimbriae *fimAICDHF,* SEF14 *sefABCD*, curli *csgDEFG*, and the plasmid encoded fimbriae *pefABCD* [29, 32, 34, 135]. The type-I fimbriae consist of *fimA*, a major subunit, and the *fimH* adhesive protein. The function of *fimH* is to determine the binding affinity of fimbriae to the target site. Although type-I fimbriae are commonly present in many Gram-negative bacteria, the structure of such proteins may vary among different species; for example the type-I *fimH* in *Salmonella* and *E. coli* are not similar and exhibit different binding specificity [29]. Such differences in the structure of type-I fimbriae implies that it may confer some beneficial functions for the virulent *Salmonella* strains.

Among the 4 kinds of fimbriae mentioned above, SEF14 is restricted to *S.* Enteritidis and the others are closely related to group D *Salmonella* [34]. SEF14 consists of the

sefABCD genes which encode 4 subunits of the SEF14 fimbriae. The *sefB* and *sefC* genes encode proteins which are homologous to the *E. coli* and *Klebsiella pneumoniae* fimbrial periplasmic chaperone proteins and fimbrial outer membrane proteins, respectively. Interestingly, *sefB* and *sefC* are not expressed if *sefA* is absent [21]. This phenomenon was discovered in 1993 and further tested in 2000. It was found that disruption of *sefA* by polar mutation results in attenuation of virulence in *Salmonella*, whereas non-polar mutation in *sefA* did not have such effect [34]. This finding shows that the subunit encoded by *sefA* is not a virulence determinant. The effect of non-polar mutation was further tested on the *sefD* subunit and found to result in significant attenuation of virulence of *Salmonella*. The expression of SEF14 fimbriae was predicted to persist if a half-function *sefA* is produces an incomplete fimbriae structure but still exhibits affinity to the target site. However, expression of SEF14 fimbriae would fail if polar mutation occurred at *sefA*. When the *sefD* gene was mutated, the SefD protein could not bind to the subunit SefC, and functional fimbriae would not be able to form (**Figure 1.3**). Unlike the SEF14 fimbriae, the long polar fimbriae *lpfABCDE* locus is a kind of specific fimbriae only found in *S*. Typhimurium. It was reported that the long polar fimbriae can facilitate the bacterial cells to adhere to M cells [47]. However, the real functions of type-I fimbriae, SEF14 fimbriae and long polar fimbriae in *Salmonella* virulence are still unclear.

Apart from being located in the chromosome, there are also plasmid-borne fimbriae genes distributed among strains of several serotypes. The *pef* operon contains plasmid-borne fimbriae genes of *Salmonella*, namely *pefBACD*. The main function of *pefBACD* is to encode fimbriae which exhibits specific binding affinity to the epithelial lining of small intestine[8].

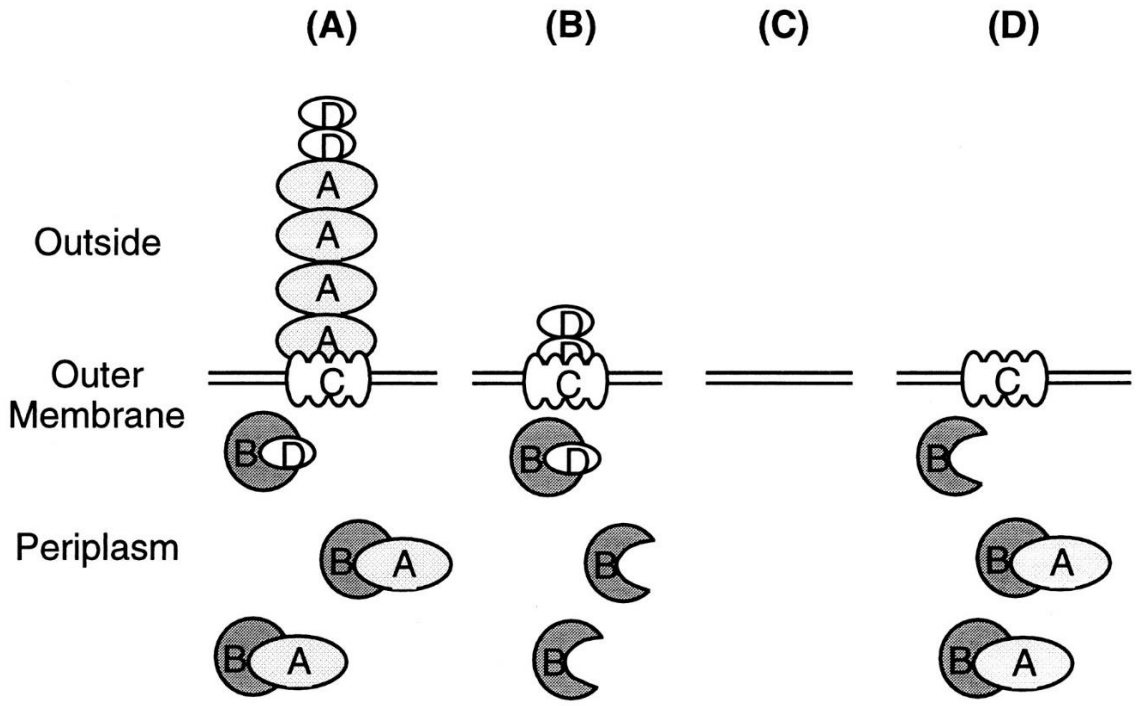

Figure 1.3. Prediction of SEF14 fimbrial subunit expression process. (A) Complete structure of SEF14 fimbriae. (B) Non-polar mutation of *sefA*, although SefA cannot bind to SefC, a short fimbrial structure can still be formed upon binding of SefD to SefC. (C) Polar mutation in *sefA* results in failure in expression of *sefABCD*. (D) Mutation in *sefD* results in failure of export of SefA to the outer-membrane. [34]

Two-Component Regulatory Systems

In order to effectively adapt the rapid changes in environmental conditions, *Salmonella* undergo frequent switching between different metabolic pathways. To facilitate rapid switching and select a correct pathway, it is necessary for bacterial cells to sense changes in the extracellular environment. *Salmonella* possesses a complex two-component system which allows it to sense such changes and translate stimuli signals into the adaptive cellular responses. Apart from the adaptive responses, expression of virulence factors is also under the regulation of two-component systems. The known virulence-regulating two-component systems in bacteria include the pleiotropic *phoP/phoQ*, *envZ/ompR*, and *barA/sirA*(*UvrY*) systems [49, 50]. These systems consist of the sensor proteins encoded by the *phoQ*, *envZ* and *barA* genes; these two component

systems are located on the surface of inner membrane so that they can sense or receive environmental signals. Once the target signals are received, their respective cytoplasmic response regulators, encoded by the *phoQ*, *ompR* and *sirA* genes respectively, will be phosphorylated to trigger numerous downstream cellular reactions [36].

In *Salmonella*, the virulence factors encoded within *Salmonella* pathogenicity island 1 and 2 were reported to be under regulated by the two-component systems *envZ/ompR* and *barA/sirA*, which play a role in post translational regulation of SPI-1 encoded regulator *hilD* and the activation of SPI-2 encoded virulence genes[35](**Figure 1.3**). Among these two-component systems, the *phoP/phoQ* system plays important roles in regulating numerous cellular activities, mediates adaptation to Mg^{2+} deficient environment, and governing virulence expression. The *phoP/phoQ* two-component system was first identified in *Salmonella enterica* serovar Typhimurium[49]. This system consists of a sensor kinase PhoQ at the inner-membrane, and a cytoplasmic regulator PhoP. It responds to the low Mg^{2+}, acidic pH environment, and some antimicrobial peptides such as polymyxin B [50]. The *phoP/phoQ* system was reported to be essential for virulence expression and survival in macrophages. A previous report showed that mutations in *phoP* resulted in reduction in virulence and survival in macrophages of *Salmonella*, but such mutation did not affect colonization of bacterial cells in the GI-tract [88]. This finding suggests that the *phoP/phoQ* two-component system plays a highly specific role in regulating the virulence level of *Salmonella* without altering its physiology. The regulatory pathway under regulation by the *phoP/phoQ* two-component system may be widely connected with expression of various virulence factors.

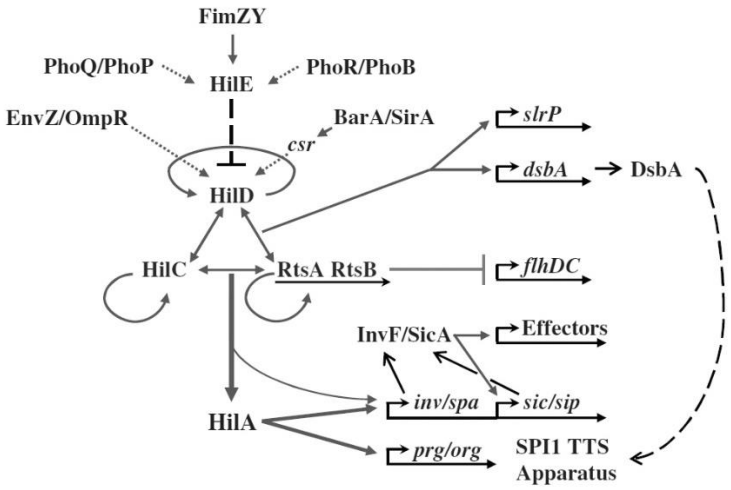

Figure 1.4. Regulatory network of SPI-encoded regulators and different two-component systems[35].

The *phoP/phoQ* system may regulate the expression of the *hilE* gene, the product of which in turn regulates expression of the *hilD* gene[35]. As *hilD* is an important regulator of various virulence factors, such regulatory system could beis regarded as a main determinant of *Salmonella* virulence phenotype. The function of *hilD* will be further discussed. The regulatory pathway of *phoP/phoQ* system is widely branched. The phosphorylated PhoP protein can repress expression of *hilA*, which is required for invasion of non-phagocytic cells and activation of expression of several virulence factors such as *ssrB*, *slyA*, *pmrA* and *rpoS* (**Figure 1.5**). Hence the *phoP/phoQ* system can regulate expression of different virulence factors at different stages of the infection process, from invasion of mucosa in GI-tract to survival in phagocytic cells[50].

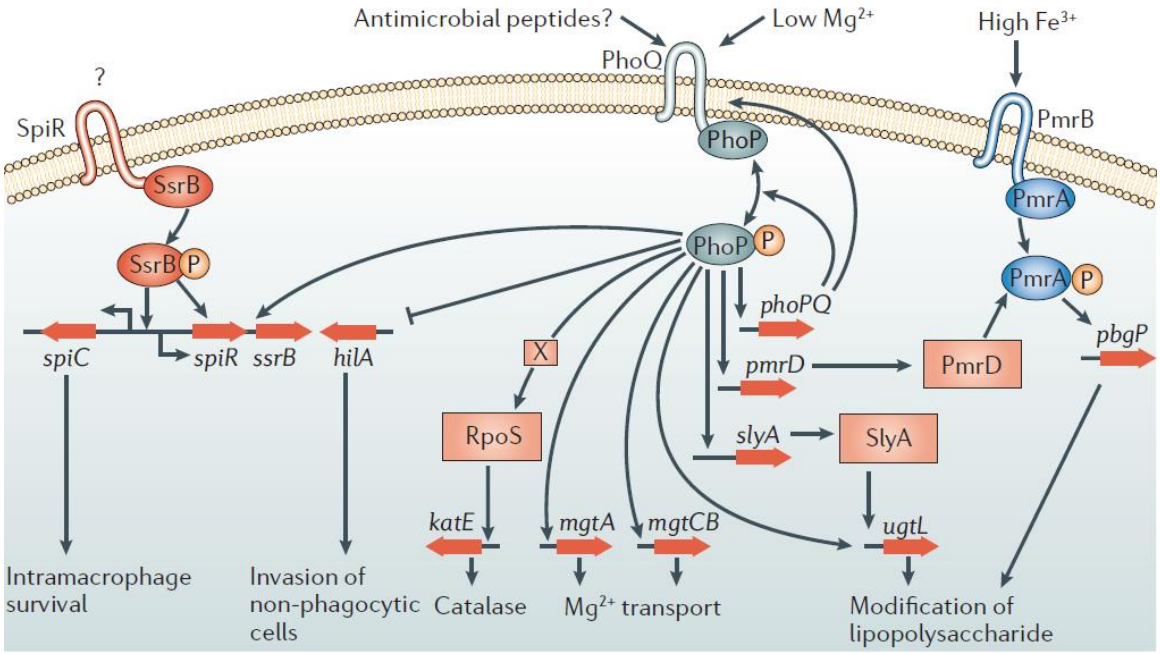

Figure 1.5. Regulatory network of the *phoP*/*phoQ* system. The virulence genes *hilA*, *ssrB*, *slyA*, *pmrA* and *rpoS* are under regulation by PhoP [50].

Salmonella Pathogenicity Island (SPI)

Salmonella is a widely distributed microbe which can survive in various environmental niches and hosts. Thus, *Salmonella* contains numerous genes which help them adapt to different habitats, such as the GI-tract of host, as well as the phagocytic cells in animal hosts. Unlike various other members of *Enterobacteriaceae* which only colonize the GI-tract of their hosts, *Salmonellae* can invade the epithelium of intestine and cause severe systemic infections.

The high invasiveness of *Salmonella* was conferred by the acquisition of numerous virulence genes which are incorporated into the chromosome during the evolution process. The virulence factors of *Salmonella* serovar Enteritidis and Typhimurium had been studied extensively. Genes that encode different virulence factors of *Salmonella* were identified and clustered at specific regions of the *Salmonella* chromosome, which is given the name of *Salmonella* pathogenicity islands. To date, 5 major pathogenicity

islands in *Salmonella enterica* have been identified: SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5 [103](**Figure 1.6**). Among these 5 pathogenicity islands, SPI-1 is the best-characterized and most-studied. SPI-1 is about 40 kilobases in size and contains numerous virulence determining factors including *invA*, *orgA*, *invG*, *prgH*, *prgK*, etc [85].

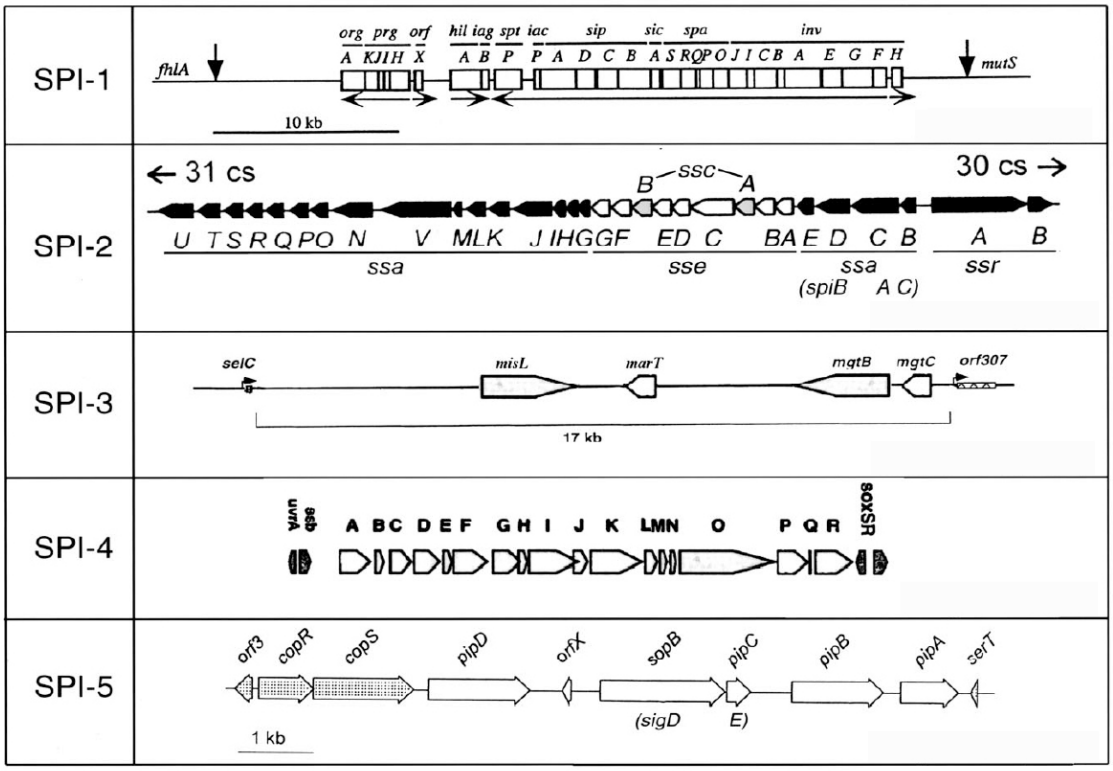

Figure 1.6. Gene map of different *Salmonella* pathogenicity islands [85].

One of the most important virulence factors in *Salmonella* is the Type-III secretion system (T3SS) which confers most of the virulence potential from the SPI cluster. This system enables bacteria to inject different virulence factors directly into the host cells cytosol by using a syringe-like secretion tool [32]. The *Salmonella* T3SS includes over 20 proteins distributed among the inner and outer membrane, periplasmic space including the peptidoglycan cell wall, as well as the extracellular space. The genes that encode T3SS are located in the chromosomal SPI-1 and SPI-2. At least 29 genes which encode different components of T3SS have been identified [85]. In addition, SPI-1 and

SPI-2 have different functions associated with pathogenesis during *Salmonella* infections. The main functions of SPI-1, including production of T3SS, allow the *Salmonella* invasion proteins to be transported across the cytoplasmic membrane of the host cell. Once the proteins have entered the host cells, they induce rearrangements of cytoskeletons, resulting in membrane ruffling and promoting internalization of *Salmonella* bacterial cells [67]. This function therefore allows *Salmonella* to induce its own uptake into the non-phagocytic cells, especially the intestinal epithelial cells [97]. In addition, it was reported that the function of SPI-1 is not confined to cell invasion, but is also cytotoxic to macrophages prior to the internalization process, causing fragmentation of chromatin, membrane blebbing, and other apoptotic damages [14].

After the *Salmonella* has successfully invaded and entered into the host, it will encounter different kinds of stress in the host body, such as deficiency of certain nutrients and chemical stress. The most challenging is the immune response of the host. As the main hosts of *Salmonella* are animals which possess a complex immune system for the defence of foreigners, survival under the attack of immune cells is a prerequisite for launching systemic infection. The SPI-2 of *Salmonella* which encodes T3SS is essential for migration through the cell membrane of phagocytic cells. Once the bacteria enter the phagocytic cells, various gene products like *ssrA*, *ssaJ*, *ssaV*, *sseA* and *sseB* from SPI-2 are expressed to facilitate proliferation of *Salmonella* [54].

To conclude, the SPI consists of various genes which encode proteins essential for invasion and systemic proliferation. Expression of these virulence related effector proteins are subjected to tight regulation. The virulence regulatory network of *Salmonella* is the subject of investigation in this study.

araC-like regulators

In *Salmonella*, expression of the SPI genes is extensively regulated by different *araC*-

like regulators, resulting in generation of a wide spectrum of virulence phenotypes among strains of different *Salmonella* serotypes. The *araC* family regulators are widely distributed in prokaryotic organisms. The family encode over 100 different products, including proteins and polypeptides that are derived from open reading frames of DNA sequences. The gene products from this family typically contain about 300 amino acids in a single molecule. Their functions commonly involve regulation of expression of genes responsible for carbon metabolism, stress response and pathogenesis. The regulatory actions of *araC* family regulators are conferred by 99 conserved amino acids next to the C-terminal. The conserved segment is consisted of DNA binding elements which can activate transcription of specific genes[44].

In *Salmonella*, the *araC* family regulators play important roles in mediating stress tolerance and adaptation to the host environment. These functions involve expression of gene products for adhesion to epithelial tissues, such as fimbriae and invasins, as well as enzymes that confer resistance to chemicals produced from host immune cells, such as ferredoxin oxidoreductase and superoxide dismutase. The known *araC* family regulators that render *Salmonella* pathogenic include *araC*, *hilD*, *hilC*, *invF* and *sprA/B*. Among these regulators, *hilD*, *hilC, sprA/B* and *invF* are located in SPI-1 and play an important role in controlling expression of SPI-1 genes. Expression of SPI-1 genes is regulated by numerous regulators. It is known that expression of the SPI-1 T3SS apparatus is induced by the *araC*-like regulator *invF*, which is in turn activated by *hilA*. Interestingly, expression of *hilA* is simultaneously regulated by the two-component system *phoP/phoQ* and the *araC*-like regulators *hilD*, *hilC* and *rtsA*. In addition, each of the *hilD*, *hilC* and *rtsA* genes is capable of self-activation and activating the transcription of *hilA* independently [45]. Such feed-forward loop regulatory network provides a precise tool to control expression of appropriate gene products at different

time points during the infection process [112].

Among various *araC* family regulators, the *hilD* gene was proposed to be the most important regulator for controlling the virulence level of *Salmonella*. It was reported that *hilD* is a dominant regulator of *hilA* expression while the effect of *hilC* and *rtsA* are more likely involved in amplification of the regulatory signal instead of serving as the main regulator [35]. Moreover, apart from activation of the SPI-1 regulon, *hilD* also induce sthe expression of SPI-2 regulon through controlling the two-component system *ssrA/B* [9]. Hence, *hilD* is an important regulator which controls the virulence of *Salmonella* in terms of the invasiveness and survival fitness of *Salmonella* in the host.

CHAPTER II-Transcriptional Regulation and Functional Characterization of the Plasmid-borne *oqxAB* Genes in *Salmonella* Typhimurium

Abstract

Co-existence of the Transferable Mechanisms of Quinolone Resistance (TMQR) genes *oqxAB* and *aac(6')-Ib-cr* in *Salmonella* Typhimurium is often associated with the expression of fluoroquinolone resistance in this important pathogen. In *Klebsiella pneumoniae*, the overexpression of chromosomal *oqxAB* operon (*oqxABR*) was highly related to its fluoroquinolone resistance phenotype[92]. However, the *Salmonella* Typhimurium carried the plasmid-borne genes (Tn*6010-oqxABR*) may not express fluoroquinolone-resistant phenotype. The molecular mechanisms underlying the fluoroquinolone susceptibility in *Salmonella* Typhimurium affected by *oqxAB* remain to be elucidated. In this study, we produced constructs carrying the *aac(6')-Ib-cr* gene and different portions of the *oqxABR* locus to investigate the role of global and local regulators in the expression of plasmid-borne *oqxAB* genes, and demonstrated that only a certain expression level of *oqxAB* could confer fluoroquinolone resistance to *Salmonella* Typhimurium. Reduced ciprofloxacin susceptibility (MIC≥1)[22] was observed only in strains carrying both *aac(6')-Ib-cr* and Tn*6010-oqxABR*. Over-expression of the plasmid-borne *oqxAB* genes does not necessarily confer ciprofloxacin resistance as a large amount of the OqxAB protein suppresses expression of the *aac(6')-Ib-cr* gene. Analysis of the Transcription Start Site sequences showed that the

expression level of *oqxR* in strains carrying *ABRp* was lower than its chromosomal (*ABRc*) counterp0art due to truncation of specific sequences, but was sufficient to induce expression of an optimal level of OqxAB that exhibits synergistic antimicrobial effect with the *aac(6')-Ib-cr* gene product to confer ciprofloxacin resistance in *S.* Typhimurium. Global transcriptional regulators in *S.* Typhimurium were also found to play a role in regulating the plasmid-borne *oqxAB* genes and fine-tuning the repressive effect of the local repressor OqxR, demonstrating that global regulatory mechanisms may control expression of plasmid-borne genes.

Introduction

Non-typhoidal *Salmonella* is the leading cause of foodborne illness worldwide, yet management of invasive infections caused by *S.* Typhimurium has become a major challenge due to the dissemination of multidrug-resistant strains[89]. Recently, OqxAB, a Resistance-Nodulation-Division-type efflux pump, has emerged as a key Transferable Mechanisms of Quinolone Resistance (TMQR) determinant amongst various members of the *Enterobacteriaceae* family, including *Salmonella* [16, 102]. The pump was found to be one of the endogenous efflux systems in *Klebsiella pneumoniae* [126]. Dissemination of the *oqxAB* gene is possibly due to acquisition of the entire locus from the chromosome of *K. pneumoniae* by IS*26* transposase, resulting in the formation of a transposon named Tn*6010,* within which the *oqxA*, *oqxB* and *oqxR* genes are flanked by two IS*26* elements[95]. This transposon was subsequently transposed into conjugative plasmids that circulate among Gram-negative bacterial pathogens. Resistance to olaquindox (substrate of OqxAB) and reduced susceptibility towards nalidixic acid and ciprofloxacin has been consistently observed in organisms harbouring the Tn*6010*-borne *oqxAB* gene, which is often accompanied by *aac(6')-Ib-cr*, another common TMQR determinants[79, 102, 128]. It remains uncertain if the

reduced fluoroquinolone susceptibility was due to *oqxAB*, *aac(6')-Ib-cr*, or the combined effect of both determinants. In *K. pneumoniae,* reduced ciprofloxacin susceptibility solely due to over-expression of *oqxAB* has just been established recently. A previous study showed that overexpressed *oqxAB* in *K. pneumoniae* resulted in increase in ciprofloxacin MIC from ≤ 0.064 μ g/mL to >3 μ g/mL[70]. As an endogenous efflux gene, the expression level of *oqxAB* in *K. pneumoniae* is regulated at both local and global levels, which is similar to the *acrAB* regulatory mechanism in other Gram-negative bacteria. Genetically, *oqxAB* in *K. pneumoniae* is flanked by two local transcriptional regulators, namely *rarA* and *oqxR*, the former is an activator for *oqxAB*, whereas the latter is a repressor [121]. Expression of *oqxAB* is also subjected to regulation by the global regulator RamA [31]. Unlike its chromosomal counterpart, *rarA* is missing from the Tn*6010-oqxAB* fragment, probably due to the excision process mediated by IS*26* [95]. It is therefore not clear how this RND-efflux pump is regulated in *Salmonella*. It has been previously shown that the plasmid-borne *oqxAB* operon is constitutively expressed in *Salmonella* Typhimurium [126]. While global regulators in *S.* Typhimurium, such as *ramA*, *marA* and *soxS*, play an integral role in regulating the host's endogenous efflux gene *acrAB* [124], whether the plasmid-borne *oqxAB* genes are also subjected to regulation by these elements, and if so, how such regulatory mechanism is controlled by cellular signals of the host, remain to be elucidated. We hypothesized that the *oqxR* gene in the plasmid-borne *oqxABR* locus may not be fully functional, resulting in over-expression of *oqxAB*. As an RND efflux pump, we also surmised that *oqxAB* may be subjected to global regulatory signals of the hosts. This study aims to evaluate the actual role of the plasmid-borne *oqxAB* genes in mediating changes in fluoroquinolone susceptibility and elucidate the mechanism by which expression of this efflux pump is regulated in *S.* Typhimurium.

Materials and Methods

Bacteria strains and vectors

Bacterial strains used in this study are listed in Table S1. *S.* Typhimurium strain 14028 (PY1, SGSC2262) was obtained from our laboratory collection. Propagation of bacteria was conducted in LB medium with/without antibiotics at 37ºC. The cloning vector used was the pACYCDuet-1 backbone (Addgene number: 71147) which contained the ampicillin resistance gene for selection purposes.

Genetic analysis of the oqxABR locus

The complete genomic sequences of *K. pneumoniae* MGH75878 (GenBank: CP000647) and plasmid sequence of pHK06-53 from *S.* Typhimurium (GenBank: KT334335) were retrieved from NCBI. The *oqxABR* locus was extracted from both and compared using ClustalW alignment. The IS element was identified and its genetic location was determined using ISfinder[111].

Generation of oqxABR operon constructs

Three constructed plasmids, namely pABRp, pABRc and pAB, which represented the plasmid-borne *oqxAB* operon, the chromosomal *oqxAB* operon and *oqxAB* alone respectively, were generated by using the modified pACYCDuet-1 as a cloning vector. The modified pACYCDuet-1 vector (pACYCDuet-AmpR) was constructed using pACYCDuet-1 (Addgene number: 71147) in which the *cmR* gene was replaced by the *AmpR* gene from pET-15b plasmid (Addgene number: 69661-3). The plasmid modification precess was done by using the Gibson Assembly Cloning Kit (New England BioLabs Inc E2611). The pACYCDuet-1 vector provided two separated multiple cloning sites for the simultaneous insertion of two gene fragments. DNA fragments of the test genes were amplified by Primestar GXL polymerase and purified by gel electrophoresis. The vector and inserted genes were double digested with

restriction enzymes and ligated by T4 ligase. pABRc was constructed by cloning the *oqxABR* operon and the 353bp upstream region of *oqxR* in *K. pneumoniae* MGH78578 (GenBank: CP000647). pABRp was constructed by cloning the *oqxABR* locus and the 84bp upstream region of *oqxR*, resembling the configuration in Tn*6010* from pHK06-53 (GenBank: KT334335). *AB* was constructed by cloning the *oqxAB* region and the 315bp sequence upstream of *oqxA* in Tn*6010* from pHK06-53. In addition, the *aac(6')-Ib-cr* gene sequence originated from pHK06-53 was inserted into pACYCDuet-AmpR, as well as the pABRp, pABRc and pAB constructs to produce four constructs: pAAC, pAAC-ABRp, pAAC-pABRc and pAAC-AB. The cloning procedures were the same as those described above. All primers used are listed in **Table 2.1**.

Antimicrobial susceptibility tests

Minimal Inhibitory Concentration (MIC) of seven antimicrobials (ciprofloxacin, gentamicin, nalidixic acid, olaquindox, cefotaxime and chloramphenicol) was determined for all test strains by using the broth microdilution method and interpreted according to the CLSI guidelines[22]. Cation-adjusted Mueller Hinton broth was used as the culture medium. *Escherichia coli* ATCC25922 was used as quality control. The test was repeated three times.

Western blotting of OqxA

The constructs of *Salmonella* Typhimurium were first streaked on LB agar plate to ensure no contamination of the stock. The single colonies from each construct were inoculated in LB broth and incubated overnight at 37°C with shaking. In the next step, the broth culture of each construct was re-inoculated to fresh LB broth and incubated at 37°C with shaking. Each construct was subjected to 3 repeated setups to ensure the consistency of result. Each test strain was grown in LB medium until the absorbance at 600nm reached 0.5. 1 ml of the culture was centrifuged and resuspended in 400µL SDS

loading buffer, then boiled for 10 minutes. Solubilised proteins were separated by SDS-PAGE and subsequently transferred to the PVDF membrane through semi-dry transfer apparatus. Western blotting was carried out by probing the membrane with rabbit anti-OqxA monoclonal antibody, followed by goat anti-rabbit IgG. The signal was visualised by the addition of HRP-substrate. *Salmonella* GAPDH-specific antibody was used as endogenous loading control.

RNA extraction and qRT-PCR

From the same batch of bacterial culture prepared for western blotting of OqxA, total RNA was extracted by the Qiagen RNeasy Protect Bacteria Minikit, followed by DNase treatment. The quality and quantity of RNA was determined by the Nanodrop spectrophotometer. One μg of total RNA was subjected to reverse transcription using the Superscript III reverse-transcriptase from Life technologies. Real-time RT-PCR reaction mixture was prepared using Life technologies SYBR Select Master mix. PCR was performed in Applied Biosystem Quant Studio 3 system. Primers used in qPCR are listed in **Table 2.1**. Melt curve analysis of PCR product was performed to ensure detection specificity. Expression levels of the test genes were normalized with the housekeeping gene which encodes the DNA gyrase subunit B. The data of gene expression level was processed using GraphPad Prism 7.00 and the statistical significance of the difference between the test samples and the control was calculated by ANOVA.

Determination of Transcription Start Site (TSS) of oqxR

TSS of the *oqxR* gene in *K. pneumoniae* and *S.* Typhimurium ST06-53 was determined by 5' Rapid Amplification of cDNA End (5'RACE), using the Invitrogen 5'RACE kit. Briefly, RNA was extracted from both strains and contaminating DNA was removed by DNase treatment, followed by conversion to cDNA by the use of primer GSP1. After

SNAP purification and TdT tailing, the dc-tailed cDNA was amplified using the abridged anchor primer and GSP2 and GSP3. The purified PCR product was TA-cloned into the pCR2.1-TOPO vector (Invitrogen™ K452001) and subsequently transformed into DH5α-T1. The cloning procedure followed the instructions provided from manufacturer. The positive-insert transformants were selected by ampicillin and Blue-white screening. The cloned sequence was confirmed by Sanger sequencing. The junction between the C tail and the start site of *oqxR* open reading frame was regarded as the transcriptional start site. The putative -10 and -35 hexamers were predicted by performing SoftBerry analysis of the intergenic region[106].

Generation of ramA and soxS knockout mutants

S. Typhimurium PY1 knockout mutants were generated by the pKD46 homologous recombination system as previously described[30]. Briefly, the helper plasmid pKD46 was electroporated into *S.* Typhimurium PY1 competent cells. Expression of λ-recombinase was induced by addition of L-arabinose. PCR product of Kanamycin resistance gene flanked by the FRT-sequence and 50bp homologous sequence were cloned into pKD4 plasmid, probed by primers listed in **Table 2.1** and were subsequently electroporated into recombinase-induced cells. Mutants were selected on LB agar plate supplemented with 50 μ g/mL kanamycin. Verification of the identity of knockout mutants was performed by PCR using primers listed in **Table 2.1**.

Electrophoretic Mobility Shift Assay (EMSA)

The open reading frame of *oqxR* was cloned into a pET28 expression vector, followed by induction of expression in *E. coli* strain BL21. The expressed protein was purified using the nickel chelated NTA column and by performing size-exclusion chromatography in an AKTA protein purification system. The promoter region of *oqxA* was amplified by PCR from *K. pneumoniae* MGH78578 using primers listed in **Table**

2.1. EMSA was performed using Gel Shift Kit 2nd Gen (Roche). Briefly, amplified DNA was labelled with DIG and incubated with the OqxR protein at room temperature for 30 minutes, followed by electrophoresis in a pre-cast 6% acrylamide non-denaturing gel with ice-cold TBE buffer. The contents were transferred to a nylon membrane by semi-dry electrotransfer system, followed by UV cross-linking. The membrane was probed against Anti-dig antibody prior to addition of the substrate for chemiluminescence detection.

Table 2.1. Primers used in this study.

Primer	Sequence (5'-3')
Knockout	
ramA-KP1	GAGCCGCTGACGAGTTTGATAGAGGGGAGAGCACGATGACT**GTGTAGGCTGGAGCTGCTTCG**
ramA-KP2	GTTGTTTTGTTTATGGTTTCTGTTGCTCGGCGCGCTGGAA**TCCATATGAATATCCTCCTTAG**
soxRS-KP1	CGCGGCGTTCAGTATTGTCAGGGATGGCACTTTGCGAAG**GTGTGTAGGCTGGAGCTGCTTCG**
soxRS-KP2	ATACAACCGTCCAGCTCATCGCGCAACGCCACCAGC**TCCATATGAATATCCTCCTTAG**
Knockout Verification	
V-ramA-F	GCGATAAGCTGTCTCACAAT
V-ramA-R	TGCTGATGGCGTTGCTCTCC
V-soxRS-F	GCGGCTAAAAATCATTGC
V-soxRS-R	CAAACCGGAACCTCCACCAC
pKD4-k1	CAGTCATAGCCGAATAGCCT
pKD4-k2	CGGTGCCCTGAATGAACTGC
Cloning	
aac-F	CGATGAATTCGTACCGGAACAACGTGATTG
aac-R	CGATAAGCTTTTAGGCATCACTGCGTGTTC
oqxAB-ABRp-F	CGATCATATGGCAGCTCCATCAGCAAAAGG
oqxAB-ABRp-R	CGATCTCGAGAAAGGCTGCCTCATCGCTAA
oqxAB-ABRc-F	CGATCATATGGCGGCCGGTTAAAAGCATC
oqxAB-ABRc-R	CGATCTCGAGGGGGGTATGTCCCCTGTTC

oqxAB-AB-F	CGATCATATGGCAGCTCCATCAGCAAAAGG
oqxAB-AB-R	CGATCTCGAGCGGTTTTTTTGTTATCTGCTGCAGG
qRT-PCR	
rrsG-F	GTTACCCGCAGAAGAAGCAC
rrsG-R	CACATCCGACTTGACAGACC
oqxA-RT-F	GCATGAAAAAGGGCCAGGTGCT
oqxA-RT-R	CGCGGGAGACGAGGTTGGTATG
oqxB-RT-F	GCGGTGGTGCTGGTAGTGATCC
oqxB-RT-R	CGCCACCACGATACCGATAGCC
oqxR-RT-F	GCAGCGAACCCGAGCTTTATCC
oqxR-RT-R	CGCGGACGGCCAAGATGAATTG
5'RACE	
OqxR-GSP1	GCGAGCGAGGACGTTTAACG
OqxR-GSP2	AGCCTGCTCTGCTTCGTCGG
OqxR-GSP3	GAAGTACCAGCAGGCGTTGG
TSS Verification	
oqxR-TSSVeriF	CTGCGGTGCCAAAAAGAACAAGA
oqxR-TSSVeriR	CCGTCACGAGTTAGCGGAACC

Table 2.2. Strains and plasmids used in this study.

Strains / plasmids	Description
K. pneumoniae MGH78578	WT strain as template
S. Typhimurium ST06-53	Clinical isolate as template, *oqxAB*+
S. Typhimurium PY1	*S.* Typhimurium 14028s Type strain
PY1/pACYC-Duet	Vector control, Amp^R
PY1/paac+AB	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxAB*[NdeI,XhoI] cloned into pACYCDuet-AmpR, Amp^R
PY1/paac+ABRc	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxABR*c[NdeI,XhoI] cloned into pACYCDuet-AmpR, Amp^R
PY1/paac+ABRp	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxABR*p[NdeI,XhoI] from ST06-53 cloned into pACYCDuet-AmpR, Amp^R
PY1Δ*ramA*	*ramA*-deleted strain, Kan^R
PY1Δ*ramA*/pACYC-Duet	Vector control, Kan^R, Amp^R
PY1Δ*ramA*/paac+AB	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxAB*[NdeI,XhoI] cloned into pACYCDuet-AmpR , Kan^R, Amp^R
PY1Δ*ramA*/paac+pABRc	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxABR*c[NdeI,XhoI] cloned into pACYCDuet-AmpR, Kan^R, Amp^R

PY1Δ*ramA*/paac+pABRp	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxABR*p[NdeI,XhoI] from ST06-53 cloned into pACYCDuet-AmpR, Kan^R, Amp^R
PY1Δ*ramR*	*ramA*-deleted strain, Kan^R
PY1Δ*ramR*/pACYC-Duet	Vector control, Kan^R, Amp^R
PY1Δ*ramR*/paac+AB	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxAB*[NdeI,XhoI] cloned into pACYCDuet-AmpR, Kan^R, Amp^R
PY1Δ*ramR*/paac+pABRc	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxABR*c[NdeI,XhoI] cloned into pACYCDuet-AmpR, Kan^R, Amp^R
PY1Δ*ramR*/paac+pABRp	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxABR*p[NdeI,XhoI] from ST06-53 cloned into pACYCDuet-AmpR, Kan^R, Amp^R
PY1Δ*soxS*	*ramA*-deleted strain, Kan^R
PY1Δ*soxS*/pACYC-Duet	Vector control, Kan^R, Amp^R
PY1Δ*soxS*/paac+AB	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxAB*[NdeI,XhoI] cloned into pACYCDuet-AmpR, Kan^R, Amp^R
PY1Δ*soxS*/paac+pABRc	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxABR*c[NdeI,XhoI] cloned into pACYCDuet-AmpR, Kan^R, Amp^R
PY1Δ*soxS*/paac+pABRp	*aac(6')-lb-cr*[EcoRI,HindIII] and *oqxABR*p[NdeI,XhoI] from ST06-53 cloned into pACYCDuet-AmpR, Kan^R, Amp^R

Results

OqxAB does not cause elevation of ciprofloxacin MIC against *Salmonella* Typhimurium

Although *oqxAB* was considered as one of the TMQR genes, its functional role in contributing to expression of fluoroquinolone resistance in *Salmonella* has not been confirmed. Previous studies showed that cloning the complete CDS of *oqxAB* from both pHK06-53 and MGH78578 into a cloning vector, followed by transformation into *E. coli*, only conferred a low MIC of 0.008μg/ml[129], suggesting that *oqxAB* is not a main determinant of fluroquinolone resistance in *Salmonella*, although it is a typical TMQR gene on its own. In this study, we cloned the full length of *oqxA* and *oqxB* gene into the pACYCDuet-AmpR vector to create p*AB*, followed by transformation into *S.* Typhimurium PY1. The ciprofloxacin (CIP) MIC of the transformant was 2-fold lower

than the vector control in *S*. Typhimurium PY1 strain, implying that *oqxAB* did not reduce ciprofloxacin susceptibility in *Salmonella* (**Table 2.3**). In *Salmonella*, *oqxAB* is commonly associated with a mobile element Tn*6010* and exists in the form of IS*26*-*oqxA*-*oqxB*-*oqxR*-IS*26*. Its original sequence from the chromosome of *K. pneumoniae* was identical to the one located in the plasmid, except that the sequence upstream of the open reading frame of *oqxR* in the *oqxABR* locus in the plasmid was truncated by the IS*26* element. Contrary to the chromosome of *K. pneumoniae* which contains a ~400bp sequence upstream of *oqxR* ahead of the next ORF, an intergenic region of only 100bp was detectable between *oqxR* and IS*26* in Tn*6010*, within which only 50bp adjacent to the *oqxR* gene are identical to the chromosomal sequence (**Figure 2.1a**). We next generated two constructs to include the full length of *oqxABR* sequences, namely pABRp and pABRc, that covered the entire *oqxABR* locus of plasmid and chromosomal origin, respectively. The CIP MIC for *S*. Typhimurium carrying pABRp was found to be 0.0625μg/ml, which was 4-fold higher than that of vector control. In contrast, the CIP MIC construct pABRc was 0.0156, which was the same level as the vector control. Such findings indicate that even the Tn*6010*-borne *oqxAB* could only slightly elevate the CIP MIC in *Salmonella* Typhimurium (**Table 2.3**).

Table 2.3. Minimal Inhibitory Concentrations (MIC) of different antimicrobials tested on strains carrying different constructs.

Strain	Constructs	MIC (μg/ml)					
		CIP	GEN	NAL	OLA	CTX	CHL
PY1	VC	0.0156	1	4	8	≤0.0625	4
PY1	AB	0.0078	2	4	16	≤0.0625	4
PY1	ABRp	0.0625	2	16	64	≤0.0625	32
PY1	ABRc	0.0156	0.5	2	8	≤0.0625	4
PY1	AAC	0.0312	2	4	16	≤0.0625	4
PY1	AAC-AB	0.0312	0.5	2	8	≤0.0625	4
PY1	AAC-ABRp	0.25	2	16	64	≤0.0625	32
PY1	AAC-ABRc	0.0625	2	8	16	≤0.0625	16
PY1Δ*ramA*	VC	0.0156	1	4	8	≤0.0625	4
PY1Δ*ramA*	AAC	0.0312	2	2	8	≤0.0625	4
PY1Δ*ramA*	AAC-AB	0.0312	0.5	2	8	≤0.0625	4
PY1Δ*ramA*	AAC-ABRp	0.25	1	16	64	≤0.0625	16
PY1Δ*ramA*	AAC-ABRc	0.0625	2	4	16	≤0.0625	16
PY1Δ*soxS*	VC	0.0156	1	4	8	≤0.0625	4
PY1Δ*soxS*	AAC	0.0312	2	4	32	≤0.0625	8
PY1Δ*soxS*	AAC-AB	0.0312	0.5	2	8	≤0.0625	4
PY1Δ*soxS*	AAC-ABRp	0.25	1	16	128	≤0.0625	16
PY1Δ*soxS*	AAC-ABRc	0.0625	2	4	16	≤0.0625	16
ATCC25922		0.0078	2	4	8	≤0.0625	4

Synergistic effect of *aac(6')-lb-cr* and *oqxAB* on mediating expression of ciprofloxacin resistance in *Salmonella*

The slight decrease in ciprofloxacin susceptibility due to acquisition of *oqxAB* in *Salmonella* as described above did not corroborate with the significantly elevated CIP MIC of *Salmonella* Typhimurium field strains, which normally exhibit CIP MIC in the range of 0.25-1μg/ml if the strains only consisted of *oqxAB* but not other resistance determinants (**Table 2.3**). We then tested if the elevated CIP MIC was contributed by *aac(6')-Ib-cr*, a common neighbouring gene of the plasmid-borne *oqxAB* gene, rather than *oqxAB* itself. We cloned the *aac(6')-Ib-cr* gene originated from pHK06-53 into a

cloning vector, pACYCDuet-AmpR, along with 213bp upstream of *aac(6')-Ib-cr* to create pAAC(6')-Ib-cr. The CIP MIC of *S.* Typhimurium PY1 carrying this construct was 0.0312μg/mL, i.e. only 2-fold increase when compared with the vector control, indicating that *aac(6')-Ib-cr* itself actually did not confer intermediate resistance (MIC≥0.5 μ g/mL) to ciprofloxacin. Such phenomenon was also reported by previous studies[39, 41]. We further cloned the *aac(6')-Ib-cr* gene into vectors that carried the gene constructs of pAB, pABRp and pABRc to generate pAAC-AB, pAAC-ABRp and pAAC-ABRc respectively. *S.* Typhimurium PY1 carrying pAAC-AB, pAAC-ABRp and pAAC-ABRc exhibited CIP MIC of 0.0312, 0.25μg/mL and 0.0625μg/mL respectively. These data indicated that *aac(6')-Ib-cr* acted synergistically with the *oqxABR* cluster in Tn*6010* to confer intermediate resistance (MIC≥0.5 μ g/mL) to ciprofloxacin. On the other hand, the chromosomal *oqxABR* cluster alone only caused a mild reduction in susceptibility to ciprofloxacin, whereas the *oqxAB* gene itself was found to have no impact on drug susceptibility even when the regulator gene *oqxR* was absent (**Table 2.3**). These findings prompted us to investigate why the plasmid containing the *oqxABR* cluster causes a significantly higher degree of elevation in MIC of CIP in the presence of *aac(6')-Ib-cr*, when compared to the chromosomal *oqxABR*, yet the *oqxAB* or *oqxABR* cluster alone has little effect on CIP MIC of *Salmonella* Typhimurium.

Regulated expression of *oqxAB* in Tn*6010* configuration confers ability to encode resistance

Based on the observations described above, sequence alignment of the plasmid-borne and chromosomal *oqxAB* gene showed that the upstream region of *oqxR* in these two clusters were different. We hypothesized that this discrepancy may alter promoter activity of *oqxR* and subsequently lead to differential expression level of this gene,

which in turn results in altered repressor activity. Since the combination of *aac(6')-Ib-cr* and *oqxABR*p only mediated expression of intermediate resistance to CIP, we therefore used pAAC-AB, pAAC-ABRp and pAAC-ABRc to carry out the following analyses. Western blotting using OqxA-specific antibody confirmed that only a trace amount of OqxA was produced in *S.* Typhimuirum PY1 carrying pAAC-ABRc, whereas a substantial level of OqxA was detectable in organisms carrying pAAC-ABRp (**Figure 2.1b**). When *oqxR* was not present, such as in the case of pAAC-AB, the expression level of the *oqxAB* genes was found to increase dramatically (**Figure 2.1b**). The data confirmed that *oqxR* indeed negatively regulated the expression level of *oqxAB*. We then further tested if the discrepancy in the level of OqxA production in pAAC-ABRp and pAAC-ABRc was due to differential expression level of *oqxR*. By performing qPCR analysis, we found that the transcription product of *oqxR* in pAAC-ABRc was about 10-fold more abundant than that of pAAC-ABRp (**Figure 2.1c**). Consistently, the level of *oqxA* transcript was found to be significantly lower in organisms carrying pAAC-ABRc when compared to those carrying pAAC-ABRp (**Figure 2.1c**). Interestingly, assessment of the degree of correlation between MIC and the expression level of OqxAB suggested that moderate expression of OqxAB in pAAC-ABRp-bearing organisms conferred the highest CIP MIC, and that lower expression level of OqxAB in strains carrying pAAC-ABRc mediated slight increase of CIP MIC. Surprisingly, high level expression of OqxAB in strains carrying the pAAC-AB construct exhibited no effect on CIP MIC. To further investigate this phenomenon, we analysed the expression level of *aac(6')-Ib-cr* in different constructs of *S.* Typhimurium PY1 by qPCR (**Figure 2.1d**). Compared with *S.* Typhimurium that carry pAAC only, the expression level of *aac(6')-Ib-cr* was reduced by 50% in strains that carried pAAC-AB, 40% in strains carrying pAAC-ABRp and 20% in strains carrying pAAC-ABRc. The significant reduction in *aac(6')-Ib-cr* expression in strains

harbouring pAAC-AB may account for the increased CIP susceptibility when compared to those with pAAC-ABRp and pAAC-ABRc. In pAAC-ABRc-bearing strains, the slight reduced expression of *aac(6')-Ib-cr* may be compensated by the addition of *oqxAB*. The expression level of OqxAB was strongly suppressed by the *oqxR* gene but resulted in only slight increment of CIP susceptibility. In pAAC-ABRp-bearing strains, the high expression level of OqxAB may overcome the effect of the slightly suppressed *aac(6')-Ib-cr* expression, and resulted in the lowest CIP sensitivity among strains carrying different constructs. The exact mechanism that causes reduction in *aac(6')-Ib-cr* expression level is not known, but could possibly due to the high fitness cost exerted by increased OqxAB production, as in the case of expression of other efflux pumps such as AcrAB in *S*. Typhimurium[96].

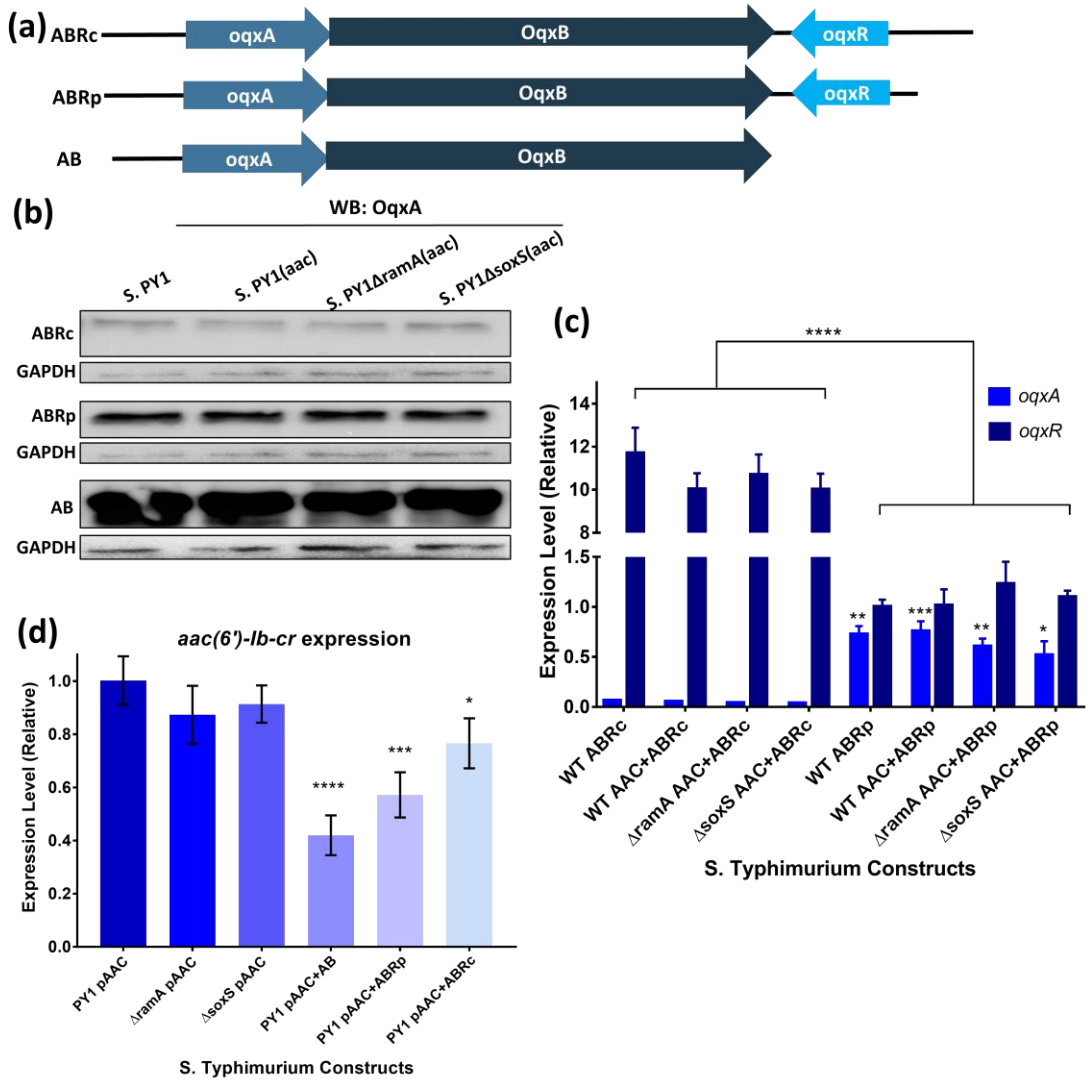

Figure 2.1. Expression of *oqxAB* and *oqxR* in different genetic constructs in *S. Typhimurium* PY1. (a) Diagrammatic illustration of three constructs generated in this study. (b) Quantification of OqxAB production in *S.* Typhimurium strains carrying different constructs by western blotting with anti-OqxA antibody. Anti-GADPH was used as normalization control. (c) Relative expression level of *oqxA* and *oqxR* in *S.* Typhimurium PY1 carrying different constructs. (d) Relative expression level of *aac(6')-Ib-cr* in *S.* Typhimurium PY1 carrying different constructs. AB, constructs carrying the *oqxAB* genes but not *oqxR*; ABRc, constructs carrying the *oqxABR* locus from *K. pneumoniae* MGH78578; ABRp, constructs carrying *oqxABR* locus from *S.* Typhimurium ST06-53; PY1, *S.* Typhimurium 14028 wild type strain; ΔramA, PY1Δ*ramA*; ΔsoxS, PY1Δ*soxS.* The data was analysed using two-way ANOVA using Prism. *, $P<0.01$; **, $P<0.001$; ***, $P<0.0001$; $P<0.00001$.

Relatively high expression level of *oqxAB* in Tn*6010* is due to partial loss of repressive function of OqxR

Like other local repressors, the repressive nature of OqxR on transcriptional regulation of *oqxAB* has been proposed to involve direct binding to the upstream region of *oqxA* [121]. In the current study, the promoter region of *oqxA* was first predicted by Softberry, followed by determination of Transcription Start Site (TSS) by 5'RACE. The identified TSS of *oqxA* was found to be 135bp upstream of the start codon, which corroborated well with the predicted -35 and -10 promoter region. This region was subsequently amplified and incubated with purified OqxR prior to EMSA analysis, which confirmed interaction between this repressor and the promoter region (**Figure 2.2a**). In view of the elevated expression of *oqxR* in pAAC-ABRc and the observation that the transcription level of *oqxA* in pAAC-ABRp also increased, it was believed that the plasmid-borne *oqxR* gene may encode a lower repressor activity due to truncation in the promoter region in Tn*6010*. To test this hypothesis, TSSs of the *oqxR* gene in the plasmid and the chromosome were determined by 5'RACE using mRNA extracted from *S.* Typhimurium ST06-53 and *K. pneumoniae* MGH78578 respectively. As expected, two different TSSs of *oqxR* could be mapped from the two strains. The TSS of *oqxR* identified from the chromosome of *K. pneumoniae* was found to be at 62bp upstream of the opening reading frame of *oqxR*; such region which has been removed and replaced by IS*26* sequences in the plasmid version. The predicted -10 and -35 promoter region also aligned well with the identified TSS (**Figure 2.2b**). Contrary to its chromosomal counterpart, transcription of *oqxR* started at 33bp inside the ORF of the plasmid-borne *oqxR* gene carried by *S.* Typhimurium. Semi-quantitative RT-PCR with primers targeting to the region of *oqxR* immediately upstream of the TSS revealed that expression was not observed in *S.* Typhimurium ST06-53, verifying the location of TSS. (**Figure 2.2c**). Taken together, our finding confirmed that truncation of the original

promoter region of *oqxR* by IS*26* in the Tn*6010*-mediated form does affect both the transcription level of mRNA and the protein sequence, which in turn alter the repressor activity of the gene product.

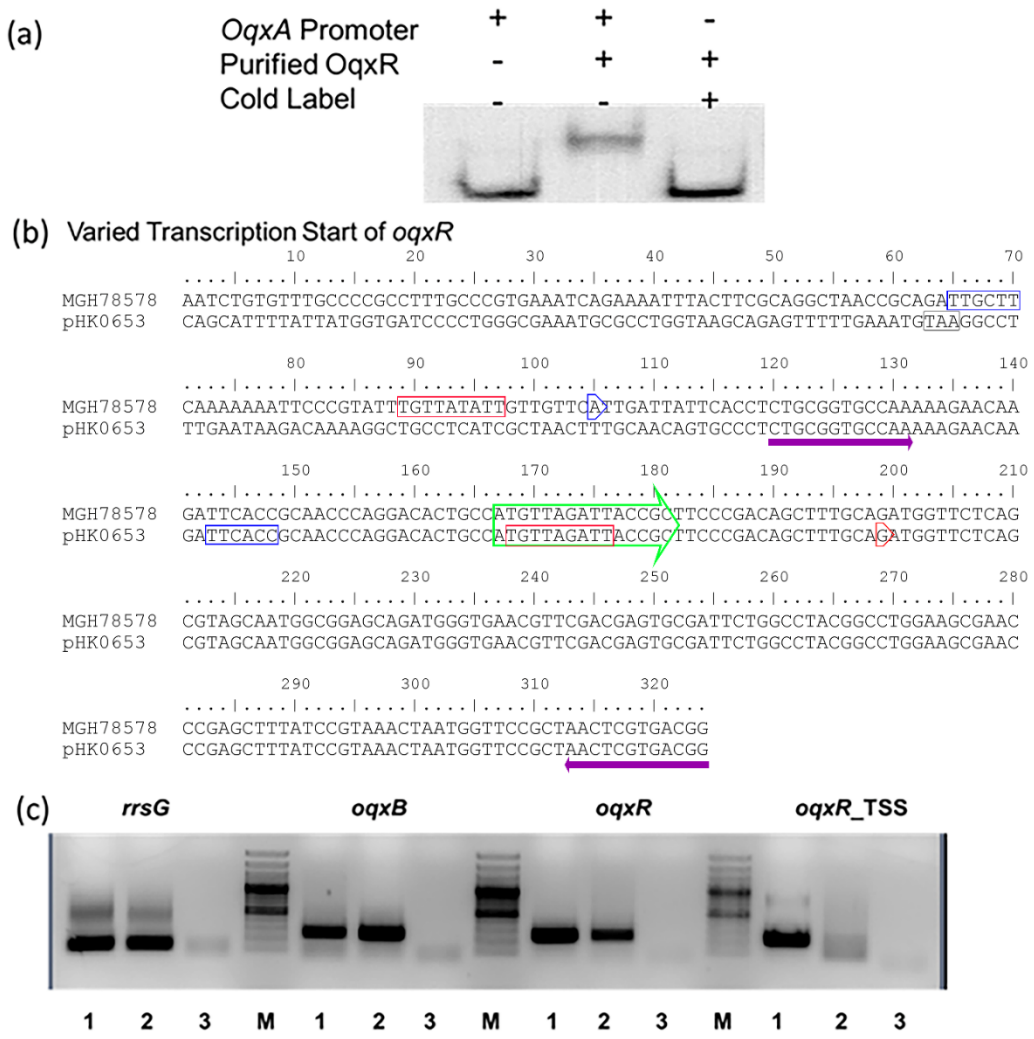

Figure 2.2. Effect of variation in *oqxR* upstream sequence on expression of the *oqxR* gene. (a) Binding of OqxR on the promoter region of *oqxAB*. (b)Transcription start site (TSS) of *oqxR* in chromosome of *K. pneumoniae* and Tn*6010*. The TSSs of *oqxR* from MGH78578 and pHK0653 are bracketed in blue arrow box and red arrow box respectively. The purple arrow denotes the binding site of primers for TSS verification. The predicted *oqxR* promoter -35 motif and -10 motif are bracketed in blue and red rectangle box respectively. The green arrow box denotes the CDS of *oqxR*. The grey rectangle box denotes the stop codon of IS26 (c) Verification of *oqxR* TSS by RT-PCR. Lane 1, *K. pneumoniae* MG78578; Lane 2, *S.* Typhimurium ST06-53; Lane 3, Negative Control.

Global regulatory genes exhibit mild effect on expression of *oqxAB* in *S.* Typhimurium

To evaluate the potential role of the global regulator genes in Tn*6010-oqxAB* expression, *S.* Typhimurium PY1 Δ*ramA* and Δ*soxS* knockout mutants were created, into which the pAAC-AB, pAAC-ABRc and pAAC-ABRp constructs were transformed. No significant changes were noted in antimicrobial susceptibility amongst *S.* Typhimurium mutants carrying various constructs. Production of OqxAB was observed in all *S.* Typhimurium PY1 test strains, and result of gene expression study showed that the *oqxAB* expression level in all transformants were similar **(Figure 2.2b, c)**, except that slight variation in *oqxA* expression level was observed in Δ*ramA* and Δ*soxS* knockout mutants carrying pAAC-ABRp. This finding indicates that *oqxAB* is constitutively expressed due to the loss of an effective local repressor *oqxR* and is subjected to transcriptional regulation by common global regulators in *S.* Typhimurium to some extent.

Discussion

The *oqxAB* and *aac(6')-Ib-cr* elements are well recognized as PMQR genes that mediated reduced susceptibility to ciprofloxacin. The high prevalence of *oqxAB*-bearing *S.* Typhimurium strains in China, where fluoroquinolones were extensively used in cattle and poultry farming, provided evidence of close relationship between *oqxAB* and fluoroquinolones resistance[116]. To further investigate the role of *oqxAB* in fluoroquinolones resistance, the *oqxAB*, *oqxABR*c and *oqxABR*p–bearing fragments were cloned into *S*. Typhimurium and subjected to MIC test. Our data for the first time confirmed that the contribution of either of these two genes to ciprofloxacin resistance is neglectable. Only a combination of these two genes exhibited the phenotype encodes by the TMQR genes. Such phenomenon aligned with the previous report that *oqxAB*-

borne plasmid-bearing *S.* Typhimurium strains may not be phenotypically resistant to ciprofloxacin, but exhibit a high prevalence in region with intensive selection pressure from fluoroquinolones usage[127]. In addition, the mRNA expression of *aac(6')-Ib-cr* appeared to be inversely proportional to the expression of *oqxAB* (**Figure 2.1d**). Interestingly, while *oqxAB* conferred a reduced ciprofloxacin susceptibility, it was observed that the growth rate of pAB and pAAC+AB transformants were significantly lower than the transformants of other constructs. Referring to the MIC result in this study, the construct pAB appeared to confer higher CIP susceptibility when compared to the vector control (**Table 2.3**). We hypothesized that, although the OqxAB efflux pump may confer certain advantage for survival of *S.* Typhimurium in the presence of fluoroquinolones, the process is associated with an extra fitness cost. It may be important for *S.* Typhimurium to control the expression level of *oqxAB* in a way to combat against antimicrobials without producing too much fitness stress. Hence, interaction between the *aac(6')-Ib-cr* and *oqxAB* gene products could affect the expression of TMQR phenotype and requires further investigation.

Another novel finding of this study is that we elucidated the mechanism of regulation of the plasmid-borne *oqxAB* efflux pumps. Our data are consistent with previous findings in that the *oqxR* gene product is a repressor of *oqxAB* in *K. pneumoniae*[121]. The result of EMSA between *oqxA* promoter and purified OqxR protein showed the molecular interaction between them. The function of OqxR can be demonstrated by the suppressed OqxAB production upon integration with *oqxR* in the pABRc construct. In line with expectation, reduction in *oqxR* expression together with increased expression of *oqxAB* in pABRp was observed, providing evidence that the *oqxR* gene in the plasmid is only weakly expressed. The GntR type transcriptional regulator OqxR shares common structures with other members in the family. It contains a typical helix-turn-

helix (HTH) domain and a ligand-binding domain[58]. Thus, it is believed that OqxR exhibits its repressive effects in a fashion like other transcription factors such as AcrR, in which its HTH domain binds to the inverted repeats (IR) nucleotide sequences located within the promoter region of *oqxAB* in the form of dimer to avoid transcription by RNA polymerase. Hence, it was the finding strongly supports the notion that decreased expression of *oqxR* in the plasmid-borne structure was due to effect of truncation of its upstream region, which results in changes in nucleotide sequences and hence a loss or damage of promoter binding sites. As a result, reduction in *oqxR* expression leads to increase in *oqxAB* expression.

In addition to local regulation, expression of OqxAB is also shown to be affected by global regulators such as *ramA* and *soxS*. Our data showed the absence of *ramA* and *soxS* resulted in slightly reduced expression of *oqxA* in Δ*ramA* and Δ*soxS* mutants (**Figure 2.1c**). Nevertheless, the effect is minimal and does not affect expression of the TMQR phenotypes. It has been reported that in *K. pneumoniae*, where endogenous *oqxAB* resides, loss of *ramR*, which resulted in over-production of RamA, would lead to over-expression of *oqxAB*[31]. Based on the partial sequences of plasmids deposited into Genbank, the TMQR determinant *oqxABR* are all found to be mediated by the transposon Tn*6010*, which was first sequenced along with an *E. coli* IncX plasmid carrying *oqxAB* in 2008 [95]. It is believed that the IS*26* transposition event captured the whole *oqxABR* locus from *K. pneumoniae*, but not the *rarA* gene located upstream of *oqxA*. A previous study has demonstrated that *rarA*, a homologue of *ramA*, may exhibit induction effect on *oqxAB* expression and subsequently confer reduced antimicrobial susceptibility in *K. pneumoniae* [121]. Considering the fact that the *rarA* product binds to the promoter region of *oqxAB*, and also its genetic homology with *ramA*, we surmise that in *Salmonella* Typhimurium, functional defect due to a lack of

the *rarA* gene can be compensated by the effect of the product of the homogenous *ramA* gene. Based on the results of the gene expression study, reduction in *oqxAB* expression was observed in strains upon in which the *ramA* or *soxS* gene was deleted, suggesting that intrinsic transcriptional factors, which have distal regulatory effects, not only regulate endogenous genes, but also those acquired through plasmid uptake or transposition. Interestingly, absence of *ramA* in *E. coli* does not hinder the substrate extrusion capacity of OqxAB, as illustrated elsewhere[15, 101], implying that this TMQR may be controlled by different regulatory mechanisms in this bacterial species.

Nevertheless, we only used *S*. Typhimurium as our model strain in this study to test for the molecular regulation of *oqxABR* operon and the effect of *ramA* and *soxS*. This limits the scope of the study and only allows us to make conclusion in *S*. Typhimurium and specific mutants. In future works, we will try to expand our study to strains of various serovars and different bacterial species in order to obtain a more complete picture on the regulation of *oqxABR* operon and other TMQR genes.

Conclusion

Our work revealed the constitutively expressed nature of the plasmid-encoded TMQR element *aac(6')-Ib-cr* and *oqxAB*. The plasmid-borne *oqxAB* gene is mainly regulated by the local repressor *oqxR*. The truncated *oqxR* sequence in plasmid-borne Tn*6010*-bearing *oqxABR* resulted in suppressed expression of OqxR and hence reduced suppression in expression of OqxAB. These events subsequently resulted in development of a ciprofloxacin resistance phenotype in *Salmonella* Typhimurium. In addition, findings in this work showed that only optimal expression of the TMQR genes *aac(6')-Ib-cr* and *oqxAB* may confer fluoroquinolone resistance in *S*. Typhimurium. Global transcriptional regulators in *S.* Typhimurium may be crucial in plasmid-borne *oqxAB* regulation and help overcome the repressive effect of the local repressor OqxR,

demonstrating that global regulatory mechanisms are capable of controlling expression of plasmid-encoded genes. Further studies are warranted to elucidate the actual events underlying the interaction between the products of *aac(6')-Ib-cr*, *oqxAB*, *oqxR* and *ramA* in *Salmonella* spp., as well as mechanisms regulating the expression of TMQR genes in other members of *Enterobacteriaceae*.

CHAPTER III-*oqxABR* Locus Reduces Virulence of *S.* Typhimurium by Inducing iNOS Expression in Macrophage to Improve Adaption to the Host

Abstract

The plasmid-mediated quinolone resistance (PMQR) efflux pump *oqxAB* was prevalent in *Salmonella* Typhimurium in the past few decades. However, the exact function of *oqxAB* gene is still under investigation. In this study, the *oqxABR* locus was cloned into *S.* Typhimurium to investigate its effect on virulence expression. The RAW264.7 infection assay and mice model were used in this study to demonstrate the virulence characteristics of *oqxABR*-bearing *S.* Typhimurium strains. The result showed that *oqxABR* could reduce virulence of *S.* Typhimurium in RAW264.7 infection model. The results of qPCR analysis showed that production of iNOS in macrophage cells was upregulated when *oqxABR* was present in *S.* Typhimurium. The result of mouse infection model also indicated that the virulence level of *S.* Typhimurium was reduced when *oqxABR* existed, but the degree of gastrointestinal tract colonisation was not affected. We hypothesize that *oqxABR* may enable *S.* Typhimurium to be more adaptive to its host by reducing its virulence level, thereby enhancing the survival fitness of the host and facilitating inter-host transmission.

Introduction

The plasmid-mediated quinolone resistance (PMQR) efflux pump-encoding gene *oqxAB* was first discovered in 2004. The gene was found to be located in the pOLA52 plasmid in an *Escherichia coli* isolate[52]. This efflux pump is structurally identical to one of the endogenous efflux system present in *Klebsiella pneumoniae*[126]. The dissemination of *oqxAB* gene was believed due to the migration of entire locus from the chromosome of *K. pneumoniae*, during which the gene is known to be flanked by the IS*26* transposase-encoding element[95]. In 2013, the *oqxAB* gene was also detected in

Salmonella spp., conferring resistance to nalidixic acid, chloramphenicol and olaquindox [131]. In the past decade, the increasing prevalence of *oqxAB*-bearing *Salmonella* Typhimurium strains has become an important public health issue worldwide. In addition, co-carriage of *aac(6')lb-cr* and *oqxAB* in *Salmonella* Typhimurium had found to be associated with fluoroquinolone resistance[127]. It was reported that a significant portion of *oqxAB*-positive *S.* Typhimurium clinical isolates were susceptible to ciprofloxacin when *aac(6')lb-cr* was not present[127]. This observation infers that the *oqxAB* efflux system may not be a key element that causes reduction in fluroquinolone susceptibility in *S.* Typhimurium. Consistently, our previous study showed that the fluoroquinolone resistance phenotype may not be expressed when only *oqxAB* is present in *S.* Typhimurium[12]. The function of *oqxAB* gene in those fluoroquinolone susceptible *S.* Typhimurium was still unclear.

Based on the concept of evolution, the high prevalence of *oqxAB* in *S.* Typhimurium was not consistent with its limited function in reducing susceptibility of fluoroquinolone type antibiotics. We hypothesized that the *oqxAB* gene may play a role in reducing the virulence of *S.* Typhimurium, hence enhancing its survival fitness and transmission between different hosts. In this study, we evaluated the effect of plasmid-borne *oqxABR* locus in mediating changes in *S.* Typhimurium virulence by performing both *in vitro* and *in vivo* assays, and elucidated the possible mechanism by which *oqxAB* optimizes the virulence level of *S.* Typhimurium.

Materials and Methods

Cloning of aac(6')-lb-cr and oqxABRp, and transformation experiments

Three plasmids, namely pAAC, pAAC-ABRp and pABRp, were constructed by respectively inserting the *aac(6')-lb-cr*, *aac(6')-lb-cr* with *oqxABR* locus and the *oqxABR* locus into the cloning vector pACYCDuet-AmpR. The pACYCDuet-AmpR

vector was constructed from pACYCDuet-1 (Addgene number: 71147), in which the *cmR* gene was replaced by the *AmpR* gene from pET-15b plasmid (Addgene number: 69661-3). Modification of the plasmid was performed by using the Gibson Assembly Cloning kit (New England BioLabs Inc E2611). The pACYCDuet-1 vector provided two separated multiple cloning sites for simultaneous insertion of two gene fragments. The *aac(6')-lb-cr* and *oqxABR* locus originated from the plasmid pHK06-53 (Genbank: KT334335). DNA fragments of the target genes were amplified by Primestar GXL polymerase (Takara Bio Inc. R050) and purified by gel electrophoresis. The corresponding primers are listed in **Table 3.1**. Both the vector and inserted genes were double digested with restriction enzymes and ligated by T4 ligase. The ligation product was chemically transformed into *E. coli* DH5α. The successful clones were selected on LB agar plate supplemented with ampicillin (100μg/mL) and confirmed by Sanger sequencing. The desired recombinant plasmids were extracted using the QIAprep Spin Miniprep Kit (QIAGEN 27106) and retransformed into *S.* Typhimurium PY1 (ATCC14028) by electroporation. The transformants were selected on LB agar plate supplemented with ampicillin (100μg/mL), followed by confirmation of their genetic identity by PCR and gel electrophoresis. The successful clones were stored in tryptic soy broth with 15% glycerol at -80℃.

Table 3.1. Primers used in this study.

Primer	Sequence (5'-3')
Cloning of *aac(6')-lb-cr* and *oqxABRp*	
oqxAB-ABRp-F	CGATCATATGGCAGCTCCATCAGCAAAAGG
oqxAB-ABRp-R	CGATCTCGAGAAAGGCTGCCTCATCGCTAA
aac-F	CGATGAATTCGTACCGGAACAACGTGATTG
aac-R	CGATAAGCTTTTAGGCATCACTGCGTGTTC
qRT-PCR	
TNF-α-qF	CCACCACGCTCTTCTGTCTACT
TNF-α-qR	AGGGTCTGGGCCATAGAACT

IL-1β-qF	TGGACCTTCCAGGATGAGGACA
IL-1β-qR	GTTCATCTCGGAGCCTGTAGTG
iNOS-qF	GGTGACCATGGAGCATCCCA
iNOS-qR	CGAACTCCAATCTCGGTGCC

Western blot of OqxA in S. Typhimurium strains carrying different constructs

Salmonella strains carrying the three constructs of pAAC, pAAC-ABRp and pABRp as described above were subjected to western blot assay to determine the expression level of OqxA in the bacterial cells. All strains were first grown in LB media at 37℃ with shaking overnight. The overnight culture of each strain was re-inoculated in fresh LB (1%) and incubated at 37℃ with shaking until the optical absorbance at 600nm reached 0.5. At the next stage, 1mL culture from each strain was collected and centrifuged at 6500xg for 3 minutes to pellet the cells. The supernatant was discarded, and the cell pellets were resuspended completely with 400μL SDS sample buffer. The cell suspensions were incubated at 100℃ for 6 minutes and then cooled to room temperature. The solubilised proteins were separated by electrophoresis in 12% acrylamide SDS gel. The separated proteins were then transferred to PVDF membrane using the Bio-Rad TransBlot® Turbo™ Transfer System. The PVDF membrane was then blocked by 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (blocking solution) for 1 hour, followed by incubation with rabbit anti-OqxA antibody in blocking solution at 4℃ overnight. The PVDF membrane was then washed with Tris-buffered saline containing 0.1% Tween-20 for 5 times, with 5 minutes intervals, followed by incubation with goat anti-rabbit IgG in blocking solution at room temperature for 1 hour. The membrane was then washed with Tris-buffered saline containing 0.1% Tween-20 for 8 times, with 5 minutes intervals. The protein bands were visualized by adding HRP-substrate; the signals were detected by the Bio-Rad ChemiDoc MP Imaging System. Broad-spectrum mouse anti-GAPDH monoclonal

antibody was used as endogenous loading control.

RAW264.7 invasion and replication assay

The virulence level of *Salmonella* Typhimurium strains containing different constructs was characterized by infecting RAW264.7 (ATCC® TIB-71™) cells and measuring the internalization and replication rate of the organisms. Single colonies of the test strains were inoculated into LB broth and incubated at 37℃ with shaking overnight. The cultures were then re-inoculated in fresh LB broth and incubated at 37℃ with shaking for 3 hours. The bacterial cells were harvested by centrifugation and washed once with phosphate buffered saline (PBS). The washed bacterial pellets were then inoculated into PBS and the optical density at 600nm (OD600) was adjusted to 1.0. The bacterial suspension was added to DMEM cell culture media to achieve a final cell concentration of 1%, and then added to RAW264.7 cells pre-coated in 24-well cell culture plate at a multiplicity of infection (MOI) of 10:1; the plates was centrifuged at 500 rpm for 5 min to synchronize the infection, followed by incubation at 37C, 5% CO_2 for 25 mins; the adhered cells were then washed twice with pre-warmed PBS and incubated with DMEM medium containing 200 μg/ml gentamicin for 1.5 h, and replaced with the medium containing 10 μg/ml of gentamicin for the rest of the experiment. The supernatant was removed at 2 and 16 h after infection, the cells were then washed twice with pre-warmed PBS and lysed with 0.2% Triton X-100. Serial dilutions of the lysates (10^{-1},10^{-2},10^{-3},10^{-4}) were plated onto XLT4 agar to enumerate the intracellular *S.* Typhimurium.

Total RNA extraction and qRT-PCR

RNA extraction from RAW264.7 macrophage

The RAW264.7 macrophage was infected by *S.* Typhimurium strains harbouring different constructs as described in RAW264.7 invasion and replication assay. After 2

hours of incubation, the culture medium was removed and immediately mixed with the RNAprotect Cell Reagent (QIAGEN 76526) for stabilization of RNA. The RAW264.7 macrophage cells were harvested and pelleted by centrifugation at 600xg for 5 minutes. The pelleted cells were subjected to RNA extraction using the RNeasy Mini Kit (QIAGEN 74106). The extraction procedure followed the instructions provided by manufacturer. The extracted RNA was then treated by DNase (Invitrogen™ AM1907) to remove any DNA contaminants. The RNA samples were stored at -80℃.

Reverse-transcription and quantitative real-time PCR (qRT-PCR)

The RNA samples were reverse transcribed to cDNA using the SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen™ 11752050). The procedure followed the instructions provided by the manufacturer. The cDNA sample was used as the template for quantitative real-time PCR. The PCR mixture was prepared by using the Luna® Universal qPCR Master Mix (New England Biolabs M3003), following a protocol provided by the manufacturer. The PCR was performed and analysed using the QuantStudio™ 3 Real-Time PCR System. The primers used are listed in **Table 3.1**.

Murine infection model

Sepsis infection model

ICR mice aged at 5 weeks were used as the host for *S.* Typhimurium infection. Each experimental group consisted of 5 mice infected by *S.* Typhimurium strains carrying different constructs. Briefly, the test strains were grown in LB agar until the optical density at 600nm reached 0.5. The bacterial cells were harvested and washed once with sterile 0.9% sodium chloride solution. The washed bacterial cells were then inoculated into 0.9% sodium chloride solution. The bacterial suspensions were injected into mice body through tail vein at the final dosage of 10^5 bacterial cells. Normal water and food

were given to each mouse during the experiment.

Gastrointestinal tract infection model

ICR mice aged at 5 weeks were used as the host for *S*. Typhimurium infection. Each experimental group consisted of 3 mice infected by *S*. Typhimurium strains carrying different constructs. Each mouse was gavage with 20 mg streptomycin daily for 3 consecutive days to eliminate intestinal microbes before starting the experiment. Culture and treatment of the test strains was the same as mentioned in sepsis infection model. The bacterial suspensions were gavage into the stomach of the mice at a final dosage of 10^7 bacterial cells. Normal water and food were given to each mouse during the experiment. The faecal samples were collected on the 2^{nd}, 4^{th}, 7^{th}, 9^{th} and 10^{th} day after infection. The mice were dissected on the 10^{th} day and the bacterial load in various organs was determined by the plate count approach.

Results

In order to confirm whether the *aac(6')-lb-cr* and *oqxABRp* genes exert extra fitness cost on *S.* Typhimurium, growth curve of strains carrying different constructs was measured (**Figure 3.1**). The results showed that the propagation rates of all constructs were similar. Therefore, the *aac(6')-lb-cr* and *oqxABRp* gene should not impose a fitness cost on *S.* Typhimurium and have no effect on the virulence level. In addition, western blot was used to confirm the production of OqxA protein in the test strains (**Figure 3.2**), with results showing that the OqxA protein was produced in strains carrying the constructs pAAC+ABRp and pABRp, but not strains harbouring the pAAC construct. This observation confirmed that the *S*. Typhimurium PY1 strains do not intrinsically carry any *oqxABR* locus. Interestingly, it was unexpected to observe higher expression level of OqxA in strains carrying pAAC+ABRp when compared to those carrying pABRp. Despite the fact that *aac(6')-lb-cr* and *oqxABR* usually coexist in the

same transferable plasmid[79, 102], *aac(6')-lb-cr* may exhibit certain regulatory effects on *oqxABR*. The mechanisms of regulation of expression of *aac(6')-lb-cr* and *oqxABR* requires further investigation.

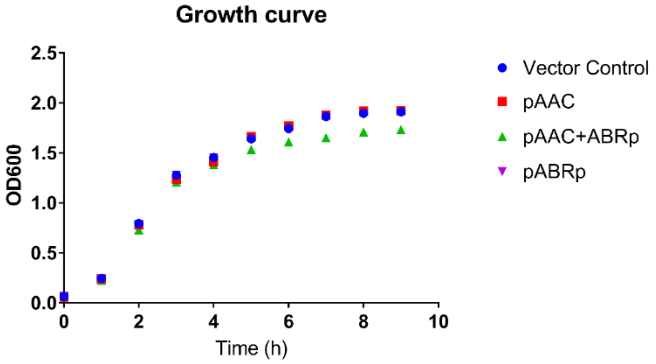

Figure 3.1. Growth curve of *S.* Typhimurium strains carrying different constructs.

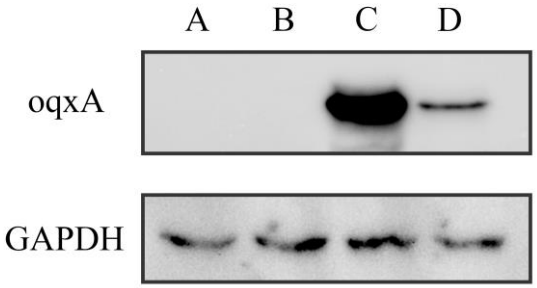

Figure 3.2. Expression level of OqxA protein in *S.* Typhimurium strains carrying different constructs as determined by western blotting. GAPDH protein was used as the loading control of the assay. Lane A, vector control; Lane B, pAAC transformed PY1. Lane C, pAAC+ABRp transformed PY1; Lane D, pABRp transformed PY1.

To investigate the effect of the *aac(6')-lb-cr* and *oqxABR* gene products on the virulence level of *S.* Typhimurium, RAW264.7 invasion and replication assay was performed to determine the intracellular survival rate of strains carrying the constructs *aac(6')-lb-cr*, *aac(6')-lb-cr+oqxABRp* and *oqxABRp* (**Figure 3.3**). As shown in **Figure 3.3a**, the macrophage invasion rate of strains carrying different constructs was about 2.5%. The result showed that existence of *aac(6')-lb-cr* or *oqxABRp* would not stimulate phagocytosis of *S.* Typhimurium in macrophages. In addition, both *aac(6')-lb-cr* or *oqxABRp* would not be beneficial or detrimental to evasion of *S.* Typhimurium from

the phagocytosis action of macrophage. The intracellular survival and replication rate were measured, and the results are shown in **Figure 3.3b**. When compared with the vector control, strain carrying the construct pAAC exhibited a similar intracellular replication rate of about 4 times during the invasion process, as recorded after 16 hours incubation. For strains carrying the construct pABRp, the intracellular replication rate during invasion decreased to 3 times, which was about 1/4 lower than the vector control. Similarly, strains carrying the construct that contained both *aac(6')-lb-cr* and *oqxABRp* exhibited an intracellular replication rate of less than 3 times during the invasion process. The result showed that *ABRp* could cause reduction in the intracellular survival fitness of *S.* Typhimurium in macrophages.

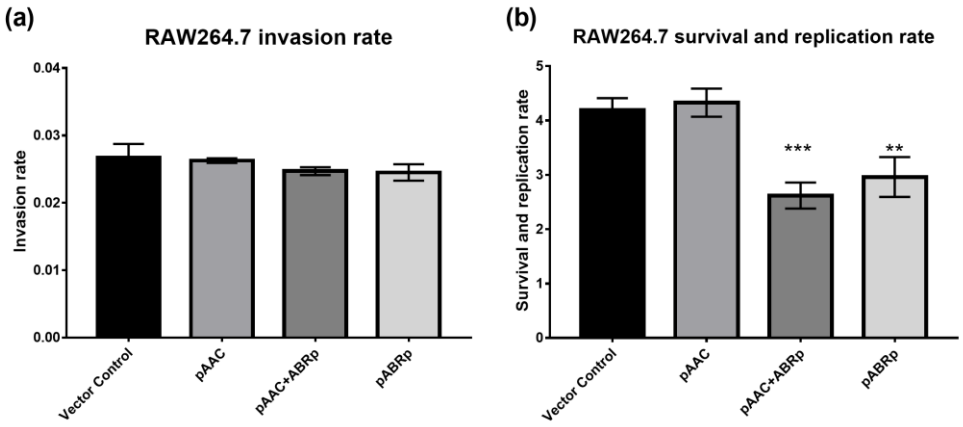

Figure 3.3. Raw264.7 invasion and replication assay. (a)RAW264.7 macrophage invasion rate of strains carrying different constructs. (b)Intracellular survival and replication rate of strains carrying different constructs in RAW264.7 macrophage.

To investigate the difference between the intracellular survival fitness of strains carrying different constructs, expression of immune factors in RAW264.7 macrophage upon infection by strains carrying different constructs was analysed (**Figure 3.4**). Compared with the no bacteria control, all the immune factors tested were found to be over-expressed when RAW264.7 macrophage cells were infected by *S.* Typhimurium.

This finding indicates that RAW264.7 macrophage cells actively respond to *S*. Typhimurium infection. The expression level of TNF-α, as shown in **Figure 3.4a**, increased about 10-fold when RAW264.7 macrophage cells were infected by the *S*. Typhimurium vector control. When infected by strains carrying the constructs pAAC, pAAC+ABRp and pABRp, the cells expressed a similar level of TNF-α as those infected by the vector control, indicating that the gene products of *aac(6')-lb-cr* or *oqxABRp* do not affect expression of TNF-α in RAW264.7 macrophage. The expression level of IL-1β in RAW264.7 macrophage increased to over 150-fold when infected by *S*. Typhimurium. Similarly, there was no significant difference in IL-1β expression upon infection by strains carrying the pAAC, pAAC+ABRp and pABRp constructs (**Figure 3.4b**). Analysis of expression level of TNF-α and IL-1β by qPCR showed that *aac(6')-lb-cr* or *oqxABRp* in *S.* Typhimurium would not stimulate the inflammatory response of macrophage cells.

Regarding iNOS expression in RAW264.7 macrophage, it was expected that the expression level of iNOS would increase upon the infection of *S*. Typhimurium, as it is an important factor that mediates production of nitrogen oxide (NO) in macrophage to eradicate intracellular pathogens[18, 53]. As shown in **Figure 3.4c**, the level of expression of iNOS RAW264.7 macrophage increased about 3-fold when infected by *S*. Typhimurium carrying the vector control and *aac(6')-lb-cr*. The expression level of iNOS further increased upon infection by *S*. Typhimurium strains carrying the pAAC+ABRp and pABRp constructs. These findings suggest that the gene product of *oqxABR* locus could induce a higher level of expression of iNOS when compared to strains without such locus. Increased expression of iNOS may increase production of intracellular nitric oxide which inflicts cellular damages in intracellular *Salmonella*[87], resulting in decreased intracellular survival and reduced rate of replication of *S*.

Typhimurium in macrophages (**Figure 3.4b**).

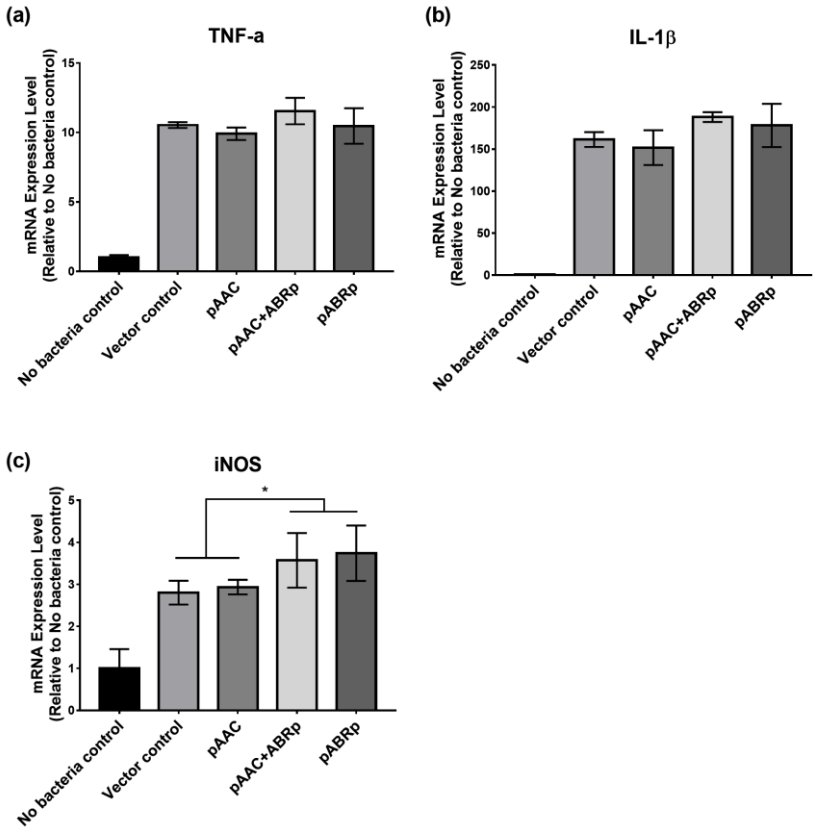

Figure 3.4. Quantitative PCR analysis of expression of immune response factors expression by RAW264.7 macrophage cells. *S.* Typhimurium strains carrying different constructs were incubated with RAW264.7 and total RNA were extracted and subjected to analysis by qRT-PCR. (a) Expression levels of TNF-α in RAW264.7 macrophage. (b) Expression levels of IL-1β in RAW264.7 macrophage. (c)Expression levels of iNOS in RAW264.7 macrophage.

In order to investigate the virulence phenotypes exhibited by strains carrying different constructs in the host body, an *in vivo* killing assay involving a murine sepsis infection model was performed. The death rate of mice in each experimental group was recorded daily (**Figure 3.5**). The results showed that a 100% mortality rate was recorded in the vector control group eight days after infection. All the mice infected by strains carrying the construct pAAC had died on the 9th day, indicating that the *aac(6')-lb-cr* gene did not affect the virulence level of *S.* Typhimurium. For mice infected by strains carrying the construct pAAC+ABRp and pABRp, up to 60% and 40% survived 18 days after

infection, respectively. All the test animals died on the 19th day for pABRp group and on the 21st day for pAAC+ABRp group (**Figure 3.5**). When strains carryng the vector control and pAAC were compared with those carrying the pAAC+ABRp and pABRp constructs, strains carrying pAAC+ABRp and pABRp required a significant longer period to kill the mice, suggesting that *oqxABR* can suppress the virulence level of *S.* Typhimurium.

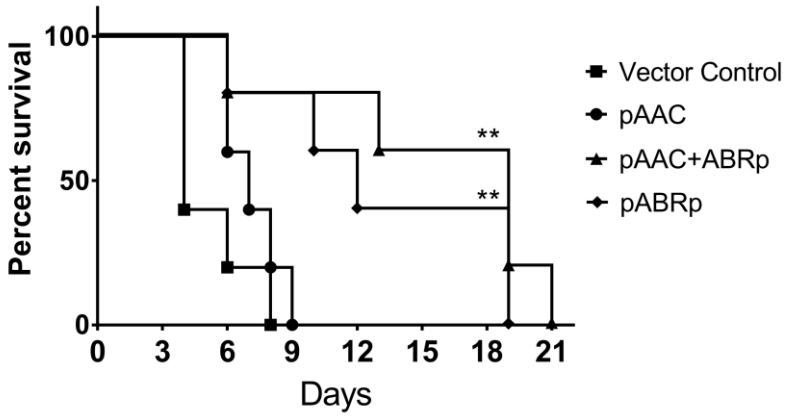

Figure 3.5. Survival rate of mice tested in a sepsis infection model. Same dosage of *Salmonella* strains carrying different constructs were injected into blood stream of ICR mice through tail vein. Percentage of survival rate of mice in each group was recorded daily.

To confirm the effect of *oqxABR* on virulence expression in *S.* Typhimurium, the mice were subjected to gastrointestinal tract infection by strains carrying the construct pABRp. The result of plate counting showed that no *S.* Typhimurium cells was present in the blood stream or the heart of the mice for both the vector control group and pABRp group (**Figure 3.6a, b**). Nevertheless, there was accumulation of *S.* Typhimurium in the lung, liver and spleen in both experimental groups (**Figure 3.6c, d, e**). However, the CFU of *S.* Typhimurium CFU in the lungs of the test mice infected by the vector control group was found to be 9-fold that of the pABRp-bearing strains (**Figure 3.6c**). The CFU data of the liver and spleen tissues showed that the number of *S.* Typhimurium in these two organs of mice infected by vector control group was double that of mice infected

by the pABRp group (**Figure 3.6d, e**). In addition, there was trace amount of *S*. Typhimurium, approximately 30 CFU/mL, found in the kidney of mice infected by the vector control group, but not the pABRp group (**Figure 3.6f**). In faecal samples, no significant difference between the two experimental groups was observed (**Figure 3.6f**). The number of *S*. Typhimurium strains carrying the vector control and the pABRp construct detectable in the faeces both ranged from 10^7 to 10^9 CFU/mL within the experimental period (**Figure 3.6g**). In the gastrointestinal tract infection model, the presence of the *oqxABR* locus in *S*. Typhimurium did not alter its ability to colonize the gastrointestinal tract of the mice. In contrast, the *oqxABR* locus appears to weaken the ability of *S*. Typhimurium into invade the internal organs of mice upon gastrointestinal tract infection.

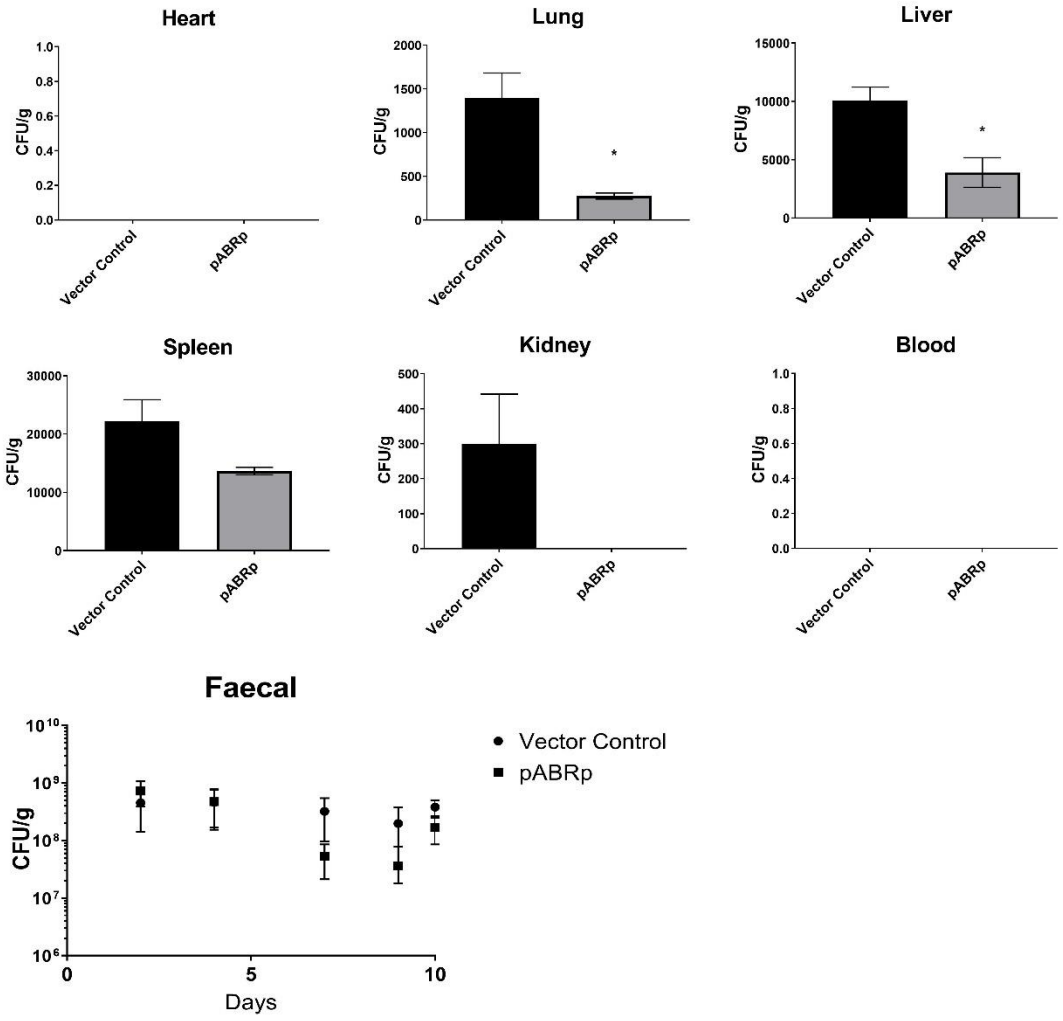

Figure 3.6. Result of gastrointestinal tract infection experiment. The number of bacteria in different specimens. (a)Bacterial count in blood. (b) Bacterial count in heart tissues. (c)Bacterial count in lung tissues. (d)Bacterial count in liver tissues. (e)Bacterial count in spleen tissues (f)Bacterial count in kidney tissues. (g)Bacterial count in faecal samples.

Results of RAW264.7 invasion and replication assay showed that the *oqxABR* locus caused reduction in intracellular survival and replication rate of *S.* Typhimurium PY1 in macrophages. Results of the sepsis infection showed that the mortality rate of mice infected with *S.* Typhimurium carrying the pAAC+ABRp and pABRp constructs decreased on Day 9 when compared with mice infected with strains carrying the vector control and the pAAC construct. Results of the mouse sepsis infection model and the

RAW264.7 macrophage infection assay indicate that the *oqxABR* locus could attenuate the intracellular survival fitness of *S*. Typhimurium in macrophage cells, resulting in more effective clearance of *S*. Typhimurium in the blood stream and reducing the chance of systemic infection. Hence, prolonged survival of mice in experimental group pAAC+ABRp and pABRp was observed. According to the result of gastrointestinal tract infection experiment, the number of invasive *S*. Typhimurium strains carrying pABRp was significantly fewer than those carrying the vector control. This finding implies that carriage of the *oqxABR* locus reduces the chance by which *S*. Typhimurium invades beyond the gastrointestinal tract and enters the blood stream of the host.

Discussion

The *aac(6')-lb-cr* and *oqxAB* genes were regarded as plasmid mediated quinolone resistant (PMQR) factors harboured by multiple bacterial species. Both genes were also reported as important determinant of fluoroquinolone resistance in *Salmonella* Typhimurium[127]. Despite the fact that fluoroquinolone is an antibiotic commonly used in clinical treatment of bacterial infections, it was found that the prevalence of *oqxAB*-bearing *E. coli* was low among clinical isolates, when compared with animal isolates in Europe where quinolones had been banned in animal feed[123]. A previous study showed that the OqxAB efflux pump was not a main determinant of fluroquinolone resistance in *S*. Typhimurium[78]. We hypothesized that the high prevalence of *oqxABR* locus in *S.* Typhimurium was not due to the selective pressure associated with the increased use of antibiotics. Instead, the OqxAB efflux pump may enable *S*. Typhimurium to colonize in the host. The result of RAW264.7 infection assay in this work showed that the intracellular survival and replication rate of *S*. Typhimurium was reduced when it was transformed with the pAAC+ABRp and pABRp construct-bearing plasmid (**Figure 3.6b**). In addition, higher expression level of OqxA

protein was detected in the strain carrying the pAAC+ABRp construct (**Figure 3.2**); a slightly lower survival fitness and replication rate in this strain, when compared to the strain carrying pABRp, was observed (**Figure 3.3b**). These findings suggest that the gene products of the *oqxABR* locus altered the ability of *S*. Typhimurium to survive in macrophage and hence facilitated eradication of intracellular *S*. Typhimurium residing in macrophage cells. Macrophage cells are an important component of the innate immune system and are widely distributed in body tissues; these cells play a role in eradicating invading pathogens non-selectively through phagocytosis and chemical digestion in phagosome[90]. *S*. Typhimurium is an opportunistic pathogen, the genome of which contains a *Salmonella* Pathogenicity Island 1 (SPI-1) that encodes gene products responsible for causing systemic infections in mice by suppressing macrophage serine synthesis, thereby promoting intracellular survival of *S*. Typhimurium[64]. To get rid of pathogens in the host body, macrophages have to express a number of gene products, including inflammatory factors (e.g. TNF-α) and lymphocyte activating factors (e.g. IL-1β), that are essential for activating the immune system of the host[122]. On the other hand, the superoxide production enzyme (iNOS) will be activated for removal of phagocytosed pathogens. When *S.* Typhimurium was phagocytosed by macrophage, the bacterial cells would be enclosed by the plasma membrane, forming phagosomes in which the invaded bacterial cells are enclosed within the cytosol. The phagosome would then maturate by fusing with lysosomes and other reactive oxygen species to kill the enclosed pathogen[74]. As a well-studied pathogen, *S*. Typhimurium is known to contain a range of pathogenicity genes that enable it to survive against the innate immune system of the host, including phagocytosis by macrophages. *Salmonella* is capable of expressing the gene products from the Type 3 secretion system (T3SS) located in the SPI-1 to prevent maturation of phagosome, forming *Salmonella* containing vacuoles (SCVs)[62, 114]. Hence, in some

circumstances *S*. Typhimurium may survive under the attack from the immune system of the host. The survived *S*. Typhimurium could replicate in SCVs and spread from the macrophages to cause serious systemic infection of the host. As shown in **Figure 3.4**, the RAW264.7 macrophage cells infected by *S*. Typhimurium carrying different constructs appeared to be over-expressed with TNF-α, IL-1β and iNOS. Moreover, the expression level of iNOS recorded when the macrophages were infected by strains carrying the constructs pAAC+ABRp and pABRp constructs was higher than those infected by strains carrying the vector control and the pAAC construct. The upregulated iNOS expression may enable macrophages to eradicate intracellular *S*. Typhimurium which carried *oqxABR* locus more efficiently. According to the result of mice sepsis infection experiment, strains bearing the *oqxABR* locus exhibited significantly reduced virulence to the mice, and extended the period of survival of the test mice by 2-fold in the experiment (**Figure 3.5**). Our results confirmed that *oqxABR* can effectively reduce the virulence of *S*. Typhimurium, possibly by impairing the survival fitness of intracellular *S*. Typhimurium. We hypothesize that the gene products from *oqxABR* locus could stimulate the production of iNOS in macrophage cells, leading to increased iNOS level in phagosomes containing engulfed *S*. Typhimurium. This event in turn results in preventing formation of matured SCVs and facilitates eradication of intracellular *Salmonella* in macrophage cells (**Figure 3.7**), thereby preventing systemic infection caused by bacterial cells released from infected macrophages. However, the mechanism of expression of OqxAB efflux pump in stimulating the host immune response is still uncleared. One of the hypotheses is that the OqxAB efflux pump may act as a kind of antigen which could be recognized by the host immune system and trigger the expression of iNOS in phagocytes. Furthermore, if our immune system is capable of recognizing the *Salmonella* OqxAB efflux pump, it is potentially possible to generate OqxAB antibody which could be a potential tool for the development of

vaccine against TMQR acquired *Salmonella* infections.

In addition, it was observed that the *S*. Typhimurium construct which carried *oqxAB* locus, with absence of *oqxR* repressor, had extremely over expressed OqxA protein and reduced the growth rate of itself. This phenomenon reflected that the gene product of *oqxAB* will generate fitness cost of the bacteria. The fitness cost can be either induced by the overproduction of OqxAB protein, or the over-expression of *oqxAB* stimulate or suppress expression of other genes in *S*. Typhimurium. Therefore, the *oqxAB* gene products may have the potential to modifying the expression profile of virulence factors in *S*. Typhimurium and help to evade from the recognition by the host immunity.

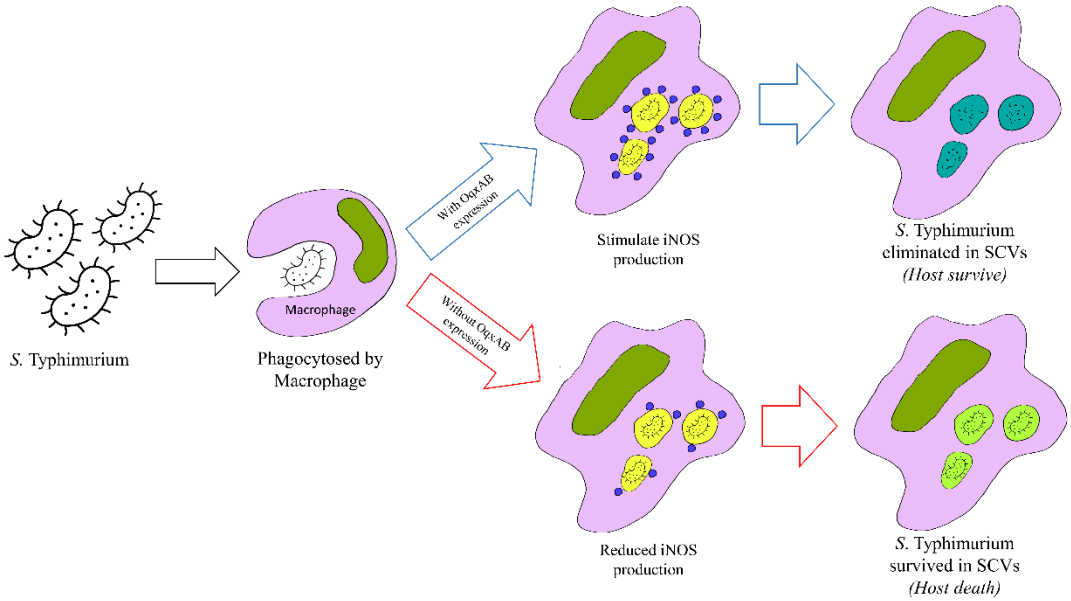

Figure 3.7. Graphical explanation of iNOS triggered by OqxAB. Expression of OqxAB in *S*. Typhimurium would stimulate production of iNOS in macrophages, which facilitating eradication of *S*. Typhimurium in *Salmonella* containing vacuole.

In the mouse gastrointestinal tract infection experiment (**Figure 3.6**), the invasion potential of strains carrying the *oqxABR*-bearing construct was reduced. Since macrophages are known to accumulate at the mucosa and lamina propria of intestine, it is important for *S*. Typhimurium to survive against the phagocytosis of macrophages before successfully invading other parts of the host body[20]. In addition, macrophage

is the resident of Kupffer cells in the liver and spleen tissue. The decreased CFU of *oqxABR*-carrying *S*. Typhimurium may be due to its reduced intracellular survival fitness in macrophage. However, the ability of *S*. Typhimurium to colonize the intestinal tract was not affected by the *oqxABR* gene (**Figure 3.6**). These findings imply that the *oqxABR* locus could prevent *S*. Typhimurium from causing systemic infection of the host, yet does not affect colonization in the gut. The *oqxABR* locus may therefore be beneficial to *S*. Typhimurium by allowing this pathogen to better adapt to the host, reducing the chance of killing the host during infection, and hence facilitating inter-host transmission. This may explained the phenomenon of the prevalence of fluroquinolone sensitive oqx*ABR* locus-bearing *S*. Typhimurium in the poultry farm[127]. For the evolution perspective, the *oqxAB* gene provides ability for *Salmonella* to coexist with their host by supressing the virulence and provide survival fitness for the bacteria itself in the host community.

Conclusion

The *oqxABR* locus of *S*. Typhimurium has limited function in reducing fluoroquinolone susceptibility. Instead, the virulence of *S*. Typhimurium can be suppressed by the *oqxABR* gene product. The reduced virulence level may be beneficial to *S*. Typhimurium as it allows the pathogen to better adapt to the host and undergo inter-host transmission. This explains why the *oqxABR* locus became prevalent among clinical *S*. Typhimurium strains without causing significant reduction in fluoroquinolone susceptibility.

CHAPTER IV-Identification of Novel Virulence Regulatory Networks in *Salmonella* Enteritidis

Abstract

Salmonella is a notorious foodborne pathogen which comprises strains that exhibit varied ability to cause human infection. To investigate molecular mechanisms underlying expression of virulence in *Salmonella* Enteritidis, a set of high and low virulence strains was subjected to comparative genomic, transcriptomic and phenotypic analysis. All test strains exhibited almost identical genetic composition, but over-expression of genes involved in various physiological functions was observable in the high virulence strains. Importantly, these genes include those responsible for maltose transport, citrate metabolism, VitB12 biosynthesis, propanediol utilization and hydrogen production, which confer the ability to utilize various organic compounds for energy production under anaerobic conditions, and hence survival in the gastrointestinal tract. Another set of genes which were over-expressed in high virulence strains, namely *tdcA*, *yaiV*, *yncC*, *yhjB*, *ramA*, *stpA* and *rnc*, were found to play a role in regulating expression of virulence genes in *Salmonella* based on analysis of gene deletion mutants: Among them, the *stpA*, *yncC* and *rnc* genes are involved in transcription regulation, hence mutations in these genes may affect expression of virulence phenotypes. Our data showed that *yhjB* and *yaiV* are important global regulator, with a role similar to that of a known virulence regulator in *Salmonella*, *hilD*. Importantly, deletion of *yhjB* or *yhjB* alone resulted in decreased invasiveness, reduced survival inside macrophages, reduced invasion to different organs, and lower mortality in animal experiments. Identification of such virulence regulatory network in *Salmonella* shall facilitate development of novel antimicrobial strategies based on suppression of virulence expression and survival fitness.

Introduction

Bacterial infection is an eternal threat to human health. Multiple approaches have been devised to combat bacterial infections since ancient times but invention and development of antibiotics that inhibit bacterial growth and attenuate bacterial virulence in the last century has been the most effective strategy. However, rapid evolution of bacteria generates mutants that exhibit novel resistant mechanisms and high-level virulence, compromising the antimicrobial effects of antibiotics. Bacterial evolution was found to be mediated by multiple factors including intrinsic mutational changes, horizontal gene transfer of extra-chromosomal genetic elements, antibiotic selection pressure and human activities that promote interaction between organisms of different bacterial species[42, 63, 76]. The combined effect of these factors renders adaptive evolution of micro-organisms highly efficient. Development of antimicrobial approaches which target newly emerged bacterial strains that exhibit novel antibiotic resistance and virulence phenotypes is necessary to safeguard human health.

Human have endured diseases caused by *Salmonella enterica* for centuries. To date, this pathogen still causes over 1 million cases of foodborne infections annually in the United States alone[1]. *Salmonella* infections are usually due to consumption of contaminated food or water. The typhoidal *Salmonellae* such as *S. Typhi* are obligate human pathogens which cause life-threatening typhoid or paratyphoid fever[43]. Unlike non-typhoidal *Salmonellae*, it is invasive and able to cause systemic infection[26]. Typhoid fever is usually endemic in developing countries. In the late 19th century, it was estimated that 16 million cases of such infection occurred annually, resulting in 600,000 deaths[25]. The latest figure provided by the World Health Organization (WHO) estimated that 11 to 20 million cases of infection occur worldwide each year, resulting in 128,000 to 161,000 deaths. The decrease in incidence of

Salmonella infection in the last century was probably due to invention of vaccine against typhoid fever.

Contrary to the small number of typhoidal serovars, non-typhoidal *Salmonella* comprise multiple serotypes. Non-typhoidal *Salmonella* infections are typically caused by intake of contaminated food or water. Unlike typhoid fever, infections due to non-typhoidal *Salmonella* are usually non-invasive. In most cases, the patients only develop self-limiting diarrhoea. Rare cases of invasive bloodstream and viscera infections mostly occur among high-risk individuals, such as immune-compromised patients[40]. Although infections of non-typhoidal *Salmonella* are generally mild, such infection is extremely common and not limited to developing countries[43]. Current data indicate that *Salmonella* is the second most common bacterial pathogen that causes food-borne infection in the United States, with the five most common serotypes being Enteritidis, Typhimurium, Newport and Javiana[11]. In the past decade, antibiotic resistant non-typhoidal *Salmonella* have become a global concern. *Salmonella enterica* contain several efflux systems, among them AcrAB-TolC is an effective efflux pump that confers resistance to a wide spectrum of antibiotics upon induction by indole, a metabolite produced by *Escherichia coli*[94]. This phenomenon suggests that gene regulation in *S. enterica* could be controlled by environmental factors, especially the chemical signals originated from other species of intestinal microbe. A previous study showed that the gene expression level of the *Salmonella* Pathogenicity Island (SPI) was up-regulated during colonization of the mucosa of intestine in new-born chicken[33], conferring high adaptability to survive and persist in the gastrointestinal tract of various animals. This ability indirectly renders *Salmonella* a successful human pathogen.

Salmonella enterica is an important food-borne pathogen and hence subjected to intensive investigation of its mechanism of virulence. In most cases, the non-typhoidal

serovars such as Typhimurium and Enteritidis cause self-limited gastroenteritis characterized by intestinal inflammation and diarrhoea. However, it was reported that up to 5% of non-typhoidal infection cases in developed countries result in invasive bacteraemia and focal systemic infections[116]. It was found that some uncommon non-typhoidal serotypes such as *S.* Choleraesuis and *S.* Dublin exhibited a higher chance of causing severe infection when compared to other non-typhoidal serotypes[65]. This observation implies that the difference in genetic environment in *Salmonella* strains of different serotypes may give rise to a difference in the degree of virulence. In particular, a reported high virulence *S.* Typhimurium ST313 strain isolated from Malawi and Kenya in Africa was found to have undergone genome degradation[71]. How the genetic environment in *Salmonella* defines virulence entails in-depth investigation.

To launch infection, bacteria must proliferate and synthesize a complex array of gene products which are essential for invasion and survival in the host[1, 9, 36, 68]. A previous study identified one common genetic difference which distinguishes between pathogenic *salmonella* and their non-pathogenic counterparts, namely carriage of the so-called *Salmonella* pathogenicity islands (SPI)[68]. The *Salmonella* pathogenicity island 1 (SPI-1) was found to be required for efficient invasion of the intestinal epithelium, and *Salmonella* pathogenicity island 2 (SPI-2) is required for proliferation and survival within macrophages, rendering the organism able to cause systemic infection[9]. The different virulence levels observable among different *Salmonella* strains was believed to be the result of genetic changes induced and selected by various environmental stresses. In this study, mechanisms underlying expression of the virulence phenotype of *Salmonella* Enteritidis was investigated by genomic and transcriptome analysis, as well as phenotypic assays, both *in vitro* and *in vivo*, with the purpose of identifying the key *Salmonella* virulence regulatory mechanisms. Findings

in this work show that the virulence level of *S*. Enteritidis is not entirely determined by carriage of specific virulence determinants, but also closely associated with the metabolic status of the organisms and expression patterns of a range of regulatory genes that have previously not been implicated in regulation of bacterial virulence. This novel view on regulation of the virulence phenotype of *S*. Enteritidis may facilitate development of new strategies to combat *Salmonella* infections.

Materials and Methods

Bacterial strains

A total of 61 *Salmonella* Enteritidis strains were included in this study, 30 of which were isolated from clinical samples in Hong Kong, three were isolated from food samples in Shenzhen, one was isolated from food sample in Hong Kong, and 29 were isolated from food samples and provided by China CDC (**Table 4.1**). The species identity of the test strains was confirmed by using the API 20E strips and by performing PCR assay targeting the *Salmonella*-specific *invA* gene. The serotypes of all test strains were determined by Oxoid™ Salmonella Test Kit.

Table 4.1. A list of 61 *S.* Enteritidis isolates collected from different sources and investigated in this study.

Isolates	Source	Location	Isolates	Source	Location
610	food	China CDC	**SE09-798**	blood	CUHK
641	food	China CDC	**SE09-2138**	blood	CUHK
642	food	China CDC	**SE09-2347**	blood	CUHK
646	food	China CDC	**SE09-237**	blood	CUHK
654	food	China CDC	**SE09-260**	blood	CUHK
662	food	China CDC	**SE09-3204**	blood	CUHK
666	food	China CDC	**SE09-3499**	blood	CUHK
667	food	China CDC	**SE09-3692**	blood	CUHK
860	food	China CDC	**SE09-709**	blood	CUHK
2842	food	China CDC	**SE11-64**	stool	CUHK
2847	food	China CDC	**SE11-65**	stool	CUHK
2865	food	China CDC	**SE11-68**	stool	CUHK
2992	food	China CDC	**SE11-69**	stool	CUHK
3006	food	China CDC	**SE11-72**	stool	CUHK
3007	food	China CDC	**SE11-73**	stool	CUHK
3008	food	China CDC	**SE11-74**	stool	CUHK
3013	food	China CDC	**SE12-1**	stool	CUHK
3015	food	China CDC	**SE12-10**	stool	CUHK
3017	food	China CDC	**SE12-2**	stool	CUHK
3018	food	China CDC	**SE12-5**	stool	CUHK

3020	food	China CDC	**SE12-6**	stool	CUHK
3021	food	China CDC	**SE12-7**	stool	CUHK
3042	food	China CDC	**SE12-8**	stool	CUHK
3046	food	China CDC	**SE12-9**	stool	CUHK
3052	food	China CDC	**SE143**	stool	CUHK
3053	food	China CDC	**SE153**	stool	CUHK
3054	food	China CDC	**SC3**	food	Shenzhen
3059	food	China CDC	**SC5**	food	Shenzhen
3064	food	China CDC	**SP2**	food	Shenzhen
SE09-1106	blood	CUHK			
SE09-1229	blood	CUHK			
SE09-1889	blood	CUHK			

DNA techniques and PCR-virulotyping

Genomic DNA of the test isolates were extracted from overnight culture and purified by using the PureLink Genomic DNA Mini Kit (Invitrogen). The purity and quantity of the purified DNA samples were determind by using the NanoDrop™ Lite Spectrophotometer (ThermoFisher Scientific) and Qubit 4 Fluorometer (ThermoFisher Scientific). Purified genomic DNA was used as template for whole genome sequencing and PCR-virulotyping. A total of 11 known virulence determinants (included *invA*) were selected for screening (**Table 4.2**). In order to characterize the virulotypes of isolates, 11 pairs of primers targeting different virulence determinants were used in PCR assay according to the previous study[60].

Standard PCR was carried out in 20 μL scaled volume. The PCR mixture contained PCR buffer and 0.1U rTaq polymerase (Takara), 10 μM of primers, 0.2 mM dNTP, 2mM $MgCl_2$ and 1 μL purified genomic DNA as template. The standard cycling conditions were 95℃ for 3 mins followed by 35 cycles of 95℃ for 30s, 55℃ for 30s, 72℃ for 1 min, and a final extension step at 72℃ for 5 min. PCR amplicons were analysed by a gel documentation system (Bio-Rad).

Table 4.2. Prevalence of known virulence genes in 61 *S.* Enteritidis isolates.

Virulence Gene	*invA*	*avrA*	*ssaQ*	*mgtC*	*siiD (spi4D)*	*sopB*	*gipA*	*sodC1*	*sopE1*	*spvC*	*bcfC*
Positive	61	61	61	59	60	57	0	58	61	53	61
Negative	0	0	0	2	1	4	61	3	0	8	0
Prevalence rate (%)	100.00	100.00	100.00	96.72	98.36	93.44%	0.00	95.08	100.00	86.89	100.00

WGS analysis of selected S. Enteritidis isolates

The WGS data were analysed and compared to the reference strain *Salmonella* Enteritidis PT4 isolate P125109 (NCBI accession NC_011294), >41.91 fold average genome coverage data were achieved, with average read length longer than 149 bp; over 89.39% of reads were found to exhibit a high degree of sequence homology to P125109 (**Table 4.3**). Each *S*. Enteritidis strain was subjected to *de novo* assembly with Celera Assembler (v8.2). Each strain could be assembled into less than 155 contigs, suggesting that the sequencing data were of high quality. The genome size was 4.7Mb in average and the CG content was between 52.09% and 52.2% (**Table 4.4**). Initial gene prediction was performed for each strain, with a predicted gene number of about 5135 for each strain (**Table 4.5**). The result of whole genome sequencing showed that slight genetic difference between clinical isolates and the food isolates existed. The high virulence *S*. Enteritidis strains were genetically identical to the low virulence strains, suggesting that the virulence level of *S*. Enteritidis was not determined by the genotype.

Table 4.3. Alignment of whole genome sequences of the test *S.* Enteritidis strains to strain P125109.

Sample	Source	Location	Number	Read Length	Base (Mbp)	Sequence Depth	Aligned %
642	food	China CDC	876,267	158:159	278.93	59.53	93.47
654	food	China CDC	679,058	156:157	213.81	45.63	93.8
3018	food	China CDC	636,439	162:163	207.92	44.37	92.67

3042	food	China CDC	729,614	159:160	234.03	49.94	94.32
3046	food	China CDC	623,684	156:157	196.38	41.91	94.8
SC3	food	Shenzhen	942,294	150:151	284.93	60.8	90.01
SE09-1106	blood	CUHK	1,560,539	149:150	469.08	100.11	93.81
SE09-1889	blood	CUHK	1,050,085	162:163	342.68	73.13	91.39
SE09-260	blood	CUHK	945,355	158:159	300.3	64.08	89.39
SE09-3692	blood	CUHK	867,186	163:164	283.81	60.57	90.61
SE11-72	stool	CUHK	1,125,032	165:166	374	79.81	89.86
SE12-5	stool	CUHK	624,037	161:162	201.78	43.06	93.32

Table 4.4. *de novo* assembly profile of the test *S.* Enteritidis strains.

Sample	Contigs #	Total Len (bp)	N50 Len (bp)	MAX Len (bp)	GC Content (%)
642	93	4,696,584	95,744	382,062	52.14
654	107	4,680,501	97,631	257,318	52.19
3018	150	4,699,326	62,951	205,405	52.2
3042	84	4,699,722	102,757	316,125	52.15
3046	100	4,641,566	82,532	196,887	52.15
SC3	78	4,709,633	101,288	524,063	52.14
SE09-1106	111	4,703,998	107,869	356,693	52.15
SE09-1889	124	4,772,272	73,438	278,408	52.09
SE09-260	152	4,712,789	52,815	295,867	52.15
SE09-3692	155	4,717,918	49,318	163,913	52.17
SE11-72	90	4,651,420	98,334	270,062	52.12
SE12-5	113	4,718,699	74,617	263,019	52.14

Table 4.5. Initial gene prediction profiles of selected *S.* Enteritidis strains.

Sample	Gene #	Total Length (bp)	Average Length(bp)	GC Content (%)
642	5137	4,115,223	801.09	53.15
654	5061	4,082,892	806.74	53.22
3018	5094	4,096,074	804.1	53.23
3042	5128	4,117,962	803.03	53.13
3046	5087	4,076,754	801.41	53.08
SC3	5181	4,134,972	798.1	53.08
SE09-1106	5112	4,104,963	803.01	53.18
SE09-1889	5238	4,164,000	794.96	53.08
SE09-260	5179	4,105,026	792.63	53.11
SE09-3692	5181	4,107,105	792.72	53.11

SE11-72	5094	4,085,550	802.03	53.08
SE12-5	5129	4,124,319	804.12	53.19

RNA extraction and RNA sequencing

The overnight culture of different bacterial strains was inoculated into fresh LB broth and allowed to growth at 37℃ with shaking until optical density reached 0.5. 5mL of log-phase culture was treated with the QIAGEN RNAprotect Bacterial Reagent. The bacterial cells were harvested by centrifugation. Total RNA was extracted by the Qiagen RNeasy Bacteria Minikit, followed by DNase treatment. The total RNA was qualified by gel electrophoresis to confirm the integrity of 23s and 16s rRNA. The ribosomal RNA in each sample was then isolated and removed using the Invitrogen™ RiboMinus™ Transcriptome Isolation Kit, bacteria. The purified RNA was subjected to RNA-seq in BGI Hong Kong Co Ltd.

Macrophage invasion and survival assay

The virulence level of *Salmonella* strains was characterized by infecting RAW 264.7 cells and determining the internalization and replication rate (**Table 4.6**). Single bacterial colonies were inoculated in LB broth and incubated at 37℃ with shaking overnight. The culture was then re-inoculated in fresh LB broth and incubated at 37℃ with shaking until the optical density of the bacterial cultures reached 0.5. The bacterial cells were harvested by centrifugation and washed once with phosphate buffered saline (PBS). The washed bacterial cells were then inoculated into PBS and the optical density was adjusted to 1.0. The bacterial suspension was added, at a rate of 1%, to DMEM cell culture medium, followed by addition to RAW 264.7 (ATCC® TIB-71™) cells pre-coated in 24-well cell culture plate with a multiplicity of infection (MOI) ratio of 10:1. The plates were then centrifuged at 500 rpm for 5 min to synchronize the infection, followed by incubation at 37C, 5% CO_2 for 25 mins. The plates were then washed twice

with pre-warmed PBS and incubated with a medium containing 200 μg/ml gentamicin for 1.5 h; the medium containing 10 μg/ml of gentamicin was then incubated for the rest of the experiment. The supernatant was removed at 2 and 16 h after infection, the cells were then washed twice with pre-warmed PBS and lysed with 0.2% Triton X-100. Serial dilutions of the lysates (10^{-1},10^{-2},10^{-3},10^{-4}) were plated onto LB agar to enumerate the amount of intracellular bacteria.

Table 4.6. The macrophage internalization and replication rate of selected *S.* Enteritidis strains which were subjected to RNA sequencing.

Strains	Source	Location	Year	Internalization rate	Replication rate
2992	food	China CDC	2012	0.0152	0.7934
3046	food	China CDC	2012	0.0135	0.0370
654	food	China CDC	2012	0.0075	1.0150
SE 12-5	stool	CUHK	2012	0.2189	7.6038
SE 11-72	stool	CUHK	2011	0.2925	4.4744
SE 09-1889	blood	CUHK	2009	0.2228	15.3199

Gene knockout experiments

Preparation of Competent cells

The knockout mutants of *Salmonella* Enteritidis were generated by using the λ-red system. Electro-competent cells of *Salmonella* were first produced by the following steps: bacteria were inoculated into LB broth and incubated at 37℃ with shaking until OD_{600} reached 0.3, followed by fast-chill in ice-water for 10 minutes. The bacterial cells were pelleted by centrifugation at 6,500xg and the supernatant was discarded. The cells were then washed with 10% ice-cold glycerol three times. The cell pellet was resuspended in 10% glycerol and stored at -80℃.

The competent cells were then transformed with pKD46 plasmid which carried the λ-red system by using electroporation. The transformed cells were recovered by adding SOC medium and incubated at 30℃ with shaking for 1 hour. The recovered cells were

spread onto LB agar supplemented with ampicillin (100μg/mL) and incubated at 30℃ overnight. Transformants carrying pKD46 were collected and inoculated into LB broth supplemented with ampicillin (100 μ g/mL) and further incubated at 30℃ with shaking. The pKD46 transformed *Salmonella* Enteritidis strains were re-inoculated into fresh LB broth supplemented with ampicillin (100μg/mL) and incubated at 30℃ with shaking until OD_{600} reached 0.3. Expression of the λ-red system was induced by 0.5% L-arabinose for 1 hour. The induced cells were made competent using the protocol described above. The competent cells were mixed with ice-chilled 10% glycerol and 100μL aliquots were prepared, flash-frozen by liquid nitrogen and stored in -80℃ for future use.

Preparation of homologous DNA fragments

A pair of primers that exhibited homology with 50bp at the 5' side at the two ends of a target gene to be deleted was used to amplify the kanamycin resistance gene in plasmid pKD4. The mutants produced are listed in **Table 4.7** and the sequence of primers used are listed in **Table 4.8**. PCR was performed by using high fidelity polymerase to ensure the integrity of the sequence in PCR products. The PCR products were purified by gel electrophoresis, followed by gel purification. The DNA fragments was dissolved in ultrapure water and stored at -20℃ until use.

Table 4.7. Gene knockout library of strain 654.

Gene category and mutant	Gene Function
Regulators	
654 ΔhilD::KanR	araC family transcriptional regulator
654 ΔyhjB::KanR	luxR family transcriptional regulator
654 ΔyncC::KanR	DNA-binding transcriptional regulator
654 ΔinvF::KanR	araC family regulatory protein
654 ΔhilC::KanR	araC family transcriptional regulator
654 ΔtdcA::KanR	DNA-binding transcriptional activator
654 ΔsprB::KanR	araC family transcriptional regulator
654 ΔstpA::KanR	DNA binding protein
654 ΔhilA::KanR	invasion protein regulator
654 ΔyaiV::KanR	DNA-binding transcriptional regulator
654 ΔaraC::KanR	araC family regulatory protein
654 ΔgerE::KanR	gerE family regulatory protein
654 ΔramA::KanR	transcriptional activator
654 ΔrecO::KanR	DNA repair protein
654 Δrnc::KanR	Ribonuclease III
B12 biosynthesis and propanediol utilization	
654 Δpdu::KanR	propanediol utilization protein
Citrate metabolism	
654 ΔcitA-css::KanR	sensor kinase, oxaloacetate decarboxylase, citrate-sodium symporter
654 ΔcitCG::KanR	[citrate (pro-3S)-lyase] ligase, citrate lyase
Cytochrome biogenesis	
654 Δnrf::KanR	cytochrome c protein, heme lyase subunit, formate-dependent nitrite reductase complex
654 Δnap::KanR	cytochrome c protein, quinol dehydrogenase, nitrate reductase, ferredoxin-type protein
654 Δccm::KanR	cytochrome c-type biogenesis protein
Fimbriae synthesis pathway	
654 ΔfimWZ::KanR	fimbriae protein transcriptional regulator, fimbriae protein
654 ΔfimAF::KanR	type-1 fimbrial protein, major pilin protein, fimbrial chaperone protein, outer membrane usher protein
Hydrogen production	
654 Δhyp::KanR	hydrogenase maturation protein, electron transport protein, formate hydrogenlyase

Maltose Transporters	
654 ΔmalEFG::KanR	maltose transporter
654 ΔmalKM::KanR	maltose/maltodextrin transporter ATP-binding protein, maltoporin, maltose regulon periplasmic protein
Nitrite reductase	
654 Δnir::KanR	nitrite transporter, nitrite reductase

Table 4.8. List of primers used for constructing knockout mutants

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
hilD	ATGGAAAATGTAACCTTTGTAAGTA	TTAATGGTTCGCCATTTTTATGAATGT
	ATAGTCATCAGCGTCCTGCCGCAGA	CGATGGCGTAGTTTTAAAATATTGTG
	ATGGGAATTAGCCATGGTCC	TAGGCTGGAGCTGCTTC
yhjB	ATGCAGGTAATCATGTTTGACAGGC	TTAATTCGATTCATTTAACATCATGGC
	AGTCAATATTTATTCATGGAATGAAA	GGCCTGGGTACGATTTTTTACATGTG
	TGGGAATTAGCCATGGTCC	TAGGCTGGAGCTGCTTC
yncC	ATGCCGGGTACGGAAAAAACACAA	TTACATGAAGTACTGATTTTTAATCG
	CATATAAGCCTGACCACACAGGTTG	TCGCTACGTTTTGTTGCAAAACGTG
	AATGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
invF	ATGTCATTTTCTGAAAGCCGACACA	TCATTTGTCTGCCAATTGAATAATATT
	ATGAAAATTGCCTGATTCAGGAAGG	TGATAATTTCCGCGGCGAAACGCGT
	ATGGGAATTAGCCATGGTCC	GTAGGCTGGAGCTGCTTC
hilC	ATGGTATTGCCTTCAATGAATAAATC	TCAATGGTTCATCGTACGCATAAAGC
	AGTTGAGGCCATTAGCAATAATCAAT	TAAGCGGTGTAATCTTAAAATGCCGT
	GGGAATTAGCCATGGTCC	GTAGGCTGGAGCTGCTTC
tdcA	ATGAATACTCTTGTTCTCCCTAAAAC	TCATTCAATTTCTATTAACTGCCTGC
	ACAGCATTTGGTGGTCTTTCAGGAA	GTCTGCATCCATTATATGATGAATGT
	TGGGAATTAGCCATGGTCC	GTAGGCTGGAGCTGCTTC
sprB	ATGAGAAATGTAATTATATACGGTATT	TTAATTCATTCCTACCGCAATCGGTA
	AACTGGACTAATTGTTATGCCCTATG	ACGCGCAATTATCGTCAGGTACAGG
	GGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
stpA	ATGAATTTGATGTTACAGAACTTAAA	TTAGATTAAGAAATCATCCAGAGATT
	TAATATCCGCACGCTGCGCGCTATAT	TCCCCGCCGCCAGCGCCTGGGCAAG
	GGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
hilA	ATGCCACATTTTAATCCTGTTCCTGT	TTACCGTAATTTAATCAAGCGGGGAT
	ATCGAATAAAAAATTCGTCTTTGAAT	CCTGTTTCCATCTTTTGAACCAAAGT
	GGGAATTAGCCATGGTCC	GTAGGCTGGAGCTGCTTC
yaiV	ATGTTATCTTTAAGCAAGCCACTACA	TTAATATTCGGAGGGCAAACGGTTA
	GGAATTCTACAGACTCGATAAATGAT	ATACTGATCAGTTTGCCTTTATTCAG

	GGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
araC	ATGCTAAAAGTATTTAATCCCTCACC	TCAATTAACATATTGATGACGAGAGG
	TGTCCAGGTGGGGAGCATTGAATGA	AAGATAAAAACGCTAAAAATTCCGG
	TGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
gerE	TTGAGATATCTGACAATGCAGTATAA	TTACGTAATATCGACTGATATATAAAA
	GAACAAAGCAAAACATATTAATATAT	CTCATTCAGCATCAAATGGATTAGTG
	GGGAATTAGCCATGGTCC	TAGGCTGGAGCTGCTTC
ramA	ATGACCATTTCCGCTCAGGTTATCGA	TCAATGCGTACGACCATGCTTTTCTT
	CACGATTGTCGAGTGGATTGATGAAT	TACGATAAGCGCCTGGCGGCAGGTG
	GGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
recO	GTGGAAGGGTGGCAGCGCGCATTTG	TTAATCTTTCTTCATTTTTACTGTGCG
	TCCTGCACAGTCGCCCCTGGAGCGA	TTTGGGCATAAATTGCCGGAACAGG
	ATGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
rnc	ATGAACCCCATCGTAATTAATCGGCT	TCATTCCAACTCCAGTTTTTTCAGCG
	TCAACGGAAGCTGGGCTACACTTTA	CCTGTTCGGCGGCAGCCTGCTCCGG
	TGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
pdu	ATGCAACAAGAAGCACTAGGAATGG	TCACTGCAGTTTGACCCCGCCTGTG
	TAGAAACCAAAGGCTTAACCGCAGC	ACCATCTTGAGTAAATGTTGTTTTGG
	ATGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
citA-css	ATGACCAACATGACCCAAGCTTCGG	TTATTCTTCTCTGGTAAAAGGGATAT
	CGACGGAGAAAAAGGGGGCTAGCG	AGAGGGTAAAAATGGTGCCAAAAG
	AATGGGAATTAGCCATGGTCC	GTGTAGGCTGGAGCTGCTTC
citCG	ATGCAGAATAGAGTGAATCTAATATT	TTACTCAGGATAGAGGGAACCGGCA
	TAAACGCATTTATTTGCAAAAGGAAT	GGAAAATGACTTAAAAACCAGGTCA
	GGGAATTAGCCATGGTCC	GTGTAGGCTGGAGCTGCTTC
nrf	ATGGCAAGGAAAACACTACGCGCAC	TTATTTCTGCCGATTTTGTAACAATTT
	GCCGTTTCTTCAGCCTTATATTTCCAT	AGTCAAATTAATGGCCTCTACCAGTG
	GGGAATTAGCCATGGTCC	TAGGCTGGAGCTGCTTC
nap	ATGGTTGATTTATCCCGTCGAAGCAT	TCAGAAACCTGGTTTAACATCACGC
	GTTGACCGGCAGTTGGCGCAACGCA	ATATCCGGCAGTTTATGCGCGATCCG
	TGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
ccm	ATGACAATGATGCTTGAAGCCAGAG	TTATTTCTCCTGCGCCAGCCGGATAC
	ATCTGTACTGCGAGCGGGACGAGAG	TGCGCTCTATCACCGCCCGCCGGGG
	ATGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
fimWZ	ATGCTGCGTATCGCTATTAAGGAACA	TTACAATAATTCGTGTGATTTGGCGT
	AAACAGTCACTTTGAGCATGGTTTAT	AATCGATAAGCTCAACGATAGAGTG
	GGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
fimAF	ATGAAACATAAATTAATGACCTCTAC	CTAATTGTAATTGATCAGGAAGGTCG

	TATTGCGAGTCTGATGTTTGTCGCAT	CATCCGCGTTAGCAAGTCCGGGCTG
	GGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
hyp	ATGACTATTTGGGAAATAAGCGAAA	CTACACTTCCCTTAACGCTCGCGCTG
	AGGCCGATTACATCGCGCAACGGCA	CGGCAATCACGCCCTGTCCAAACGG
	ATGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
malEFG	ATGAAGATTAAAACTGGCGTAGGCA	TTAACCTTTCACACCCCCAGCCGTC
	TCCTCGCATTATCCGCACTTACGACA	AGGCCGTTGACCAGCCAACGTTGCG
	TGGGAATTAGCCATGGTCC	GTGTAGGCTGGAGCTGCTTC
malKM	ATGGCGAGCGTACAGCTACGAAATG	TTACCCCTTGCCTTTTACACTGCTGA
	TAACGAAAGCCTGGGGTGACGTGGT	TAAAGGTGGAACGGGCAGATGTCGG
	ATGGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC
nir	ATGAGCAAAGTCAGACTCGCTATTAT	TTAATGGTTGGCTGTAACCTTAATTT
	CGGTAATGGTATGGTCGGCCACCGAT	GGTTGGTTTTTTGCGGAACAGGACG
	GGGAATTAGCCATGGTCC	TGTAGGCTGGAGCTGCTTC

Transformation of homologous DNA fragments and selection of knockout mutants

The competent cells prepared from the previous steps were thawed slowly on ice; 5μL of homologous DNA fragments were added to the thawed competent cells and mixed gently. Electroporation (E=18kV/cm) was performed upon addition of prewarmed SOC medium. The transformants were recovered at 37℃ with shaking for 1 hour. The recovered cells were spread onto agar plates supplemented with kanamycin (50μg/mL), followed by incubation at 37℃ overnight. The single colonies were inoculated into LB broth supplemented with kanamycin (50μg/mL) and incubated at 37℃ with shaking overnight. Deletion of the target gene was confirmed by Sanger sequencing. The mutants were kept viable by storing at -80℃ with 15% glycerol.

RNA extraction and qRT-PCR

The overnight culture of the test strains was first re-inoculated into fresh LB broth and allowed to grow at 37℃ with shaking until optical density reached 0.5; 1mL of log-phase culture was then treated with the QIAGEN RNAprotect Bacterial Reagent. The bacterial cells were harvested by centrifugation. Total RNA was extracted by the Qiagen

RNeasy Bacteria Minikit, followed by DNase treatment. The quality and quantity of RNA was determined by using the Nanodrop spectrophotometer. One μg of total RNA was subjected to reverse transcription using Life technologies Superscript III reverse-transcriptase. Real-time RT-PCR was performed by using the Applied Biosystem Quant Studio 3 and the Life technologies SYBR Select Master mix. Primers used in qPCR are listed in Table S9. Melt curve analysis of PCR product was performed to ensure specificity. Expression levels of the test genes were normalized with housekeeping gene that encodes the DNA gyrase subunit B. The primers used for each qPCR reaction are listed in **Table 4.9**.

Table 4.9. List of primers used in qRT-PCR.

Gene	Forward Primer	Reverse Primer
gyrB	CAGATCTACGAGCACGGCGT	AGTGAAGGTTTCGTGGCTCGG
invF	CGCACCAGTATCAGGAGACC	TCCACTAATCCTGCGCCATC
sprB	CGAGAGAAGCTCCGTCTTCCG	ACAATACCTCTTCCGGCGTCT
tdcA	GAGGTATTTCCAAAAGCGCAGGTATCC	CGATAGCAAAGTCCAGCCGCC
yaiV	GCAGGCCCCCTTTATTTTCGGTT	AACCGCTCTCCGCAATTAATTT
hilD	CGTGACGCTTGAAGAGGTCAATGG	GTGCATAGAGAGCGCCAAGTCG
hilC	CATGCGGACTTGTTGCCAGG	CTCACATTACTACAAACCCGTGGACA
hilA	CCCGCTTCTCTCTTGCAAAACAAAATTG	ATCTGCTTTGTGTCCCAGCGAAG
yncC	TGGCGGTGGTCGCCGCTG	CCACTTTCCAGCGCCTGTTGAA
yhjB	GGATGGCAGTCAAAAGTGGCTGCA	GCGCAAGGACAAAGGTTTCGGC
ramA	TATTCCAAGTGGCACCTGCAGCGC	AGCTTCCGTTCACGCACGT
stpA	GACGCGAAGAAGAAGAATTGCAGC	CTTCCGGGTTAATACCGTCTGCTT
rnc	AAAAAGCGGCGGATTCCGTCG	GGATTAACTGCTCGACGGTCTGG
invC	AACGGAGGCGGATGTCTTTGT	AGCGCGCAACATATCCACGAA
sopE	GGCGCTATGTCGATTCCTTTGC	CAGCCAGACCCGTGAAGCTAT
bcfC	CGAGAAGGGCGGCTGAAGTAT	TTGTGTTCCGCCATACAGCGT
invA	GGCGAGCAGCCGCTTAGTATT	CCGGCTCTTCGGCACAAGTAA
ssaB	GTGATGGCCCAGCCATGGAG	GGCATCCAGCATCAAGGCATG
sscA	GCGCAGCGCGACTTTTTCAAT	TTTGAGCCTGGCAGCATTCCC
ssaV	AGCCCGACGTTACATTCTGCC	GAGGGACGCCGGTATCCTCAA
ssaU	GTACTTTTCGGGCGCTACCGT	GCCATCACCCCAACCCATAACC
ssaQ	ATGGCCAACGGGTTTTTTGCG	CGTAAGCTGGCACCAGCCTAA

sopB	ACGCTTTTTTGCGGGATACGT	GGCTTTGTTTTCAGCGCCGAC
siiA	ACGAAAGTAATCCGTGGCCT	TGGCTCCGCTATTTTGAGCA
siiD	ACCGGAGGAACAATTCAGCG	GCTTTGTCCACGCCTTTCAT
siiE	TATTGCCCCTGTACCTCCGA	GTAGACTTTGGCTCTGCCGT
fimA	GGTGACGCTGGGTCAATACCG	GCCACTTTCGGATCGCAGTCA
fimC	CAGCGAGCCCAAAAGCGAAAA	GACCGACGGGATGGCTTTCAC
fimD	TCCTACCTCTTCCAGCGGCGA	CTCACGCTGCAATAGCGGAAC
fimZ	GAATTACCGGGCACCGACGG	CCGCTCTTATTGCTCTTCCGGC
malK	CGGAGCCGCGTGTGTTTTTG	GCGGCCCAGACGTTTATGCA
malG	CCAGATGTTTCCGGCGGTTTT	GCCGCCATGCGTATTCAGACC
citF2	GCAGGCGGCTAATGTGATCGA	TTCCAGGAAACGGGTGACCGC
citC2	ATCGGCGCCATCGTCATGAAC	AGATGCAACCAGTCACAGGCC
pduA	TTAACCGCAGCCATAGAGGCCG	AGCCCGGAGCCAATCTTTTCAT
pduD	TTTTGCGCGAAGTCATTGCCGG	TTACCTTCCACGGCGACGAACG
pduP	GCGACCCAGCAGATGATGGC	CCGCTCTTCATGCCCATTGC
napB	GGAAGGGGCGATTCGTATGCC	TCGACGCTGTGCGGGATCAT
ccmH	ACGGCAACTTCGTCACCTACG	GGCAACGATTATCCACCCGCC
nrfA	GCGGGACGATTTGATCAGCAATC	ATCCCACGGGAACTTAACGGC
hycA	CGCGTTGGCGGATGGTTTTAAC	GAGCTGCGCCTGTGCAATCAA
hycF	TTCTGCGGTCGCTGCGAAGAA	AAGCGGGACTGTTGCAGGAAG
hydN	TCGCCAGTGTGAAGACGCGC	GCAGCGTTCCTGCATCACAT
nirB	TGAACGCCATCGAACTGCCG	CGGCAGTTTATCCACGCCGA
nirC	ATTTGACCGTGGCTGATGGT	GTCGGTCTCGGCATCATTCT

Murine infection assay

Sepsis infection model

ICR mice aged 5 weeks were used as the host for *S.* Enteritidis infection. Each experimental group consisting of 5 mice was infected by different mutants of *S.* Enteritidis. Briefly, S. Enteritidis mutants were first grown in LB broth until the optical density at 600nm reached 0.5. The bacterial cells were harvested and washed once with sterile 0.9% sodium chloride solution. The washed bacterial cells were then inoculated into 0.9% sodium chloride solution. The bacterial suspensions were injected into the mice through tail vein at the final dosage of 10^5 bacterial cells. Water and food were

given to each mouse during the experiment. The death rate of mice in each experimental group was recorded at 12-hour intervals.

Gastrointestinal tract infection model

ICR mice aged 5 weeks were used as the host for *S.* Enteritidis infection. Each experimental group consisting of 6 mice was infected by different mutants of *S.* Enteritidis. Each mouse was gavage with 20 mg streptomycin daily for 3 consecutive days to eliminate intestinal microbes before the start of the experiment. Growth and treatment of *S.* Enteritidis mutants was the same as mentioned in sepsis infection model. The bacterial suspensions were gavage into the stomach of the mice at the final dosage of 10^7 bacterial cells. Normal water and food were given to each mouse during the experiment. The faecal samples were collected on the 1^{st}, 2^{nd}, 3^{rd}, 5^{th}, 7^{th}, 8^{th},10^{th}, 12^{th} and 14^{th} day after infection. The mice were dissected on the 7^{th} and 14^{th} day (3 mice on each day) and the bacterial load in various organs was determined by the plate-count approach. All animal experiments were approved by the Animal Research Ethics Sub-committee, City University of Hong Kong.

Results

Difference in virulence levels of foodborne and clinical *S.* Enteritidis strains is not due to genetic variations

The virulence level of 61 *S.* Enteritidis strains (**Table 4.1**) recovered from food and clinical samples, most of which were genetically unrelated according to PFGE analysis (**Figure 4.1**), were investigated by performing invasion assays and macrophage survival assays. The internalization rates were found to range from 0.0057 (low) to 0.1798 (high) for food isolates, and from 0.0446 (low) to 15.3199 (high) for clinical isolates. The survival fitness was found to be in the range of 0.0099 (low) to 75.5618 (high) for food isolates and 0.0013 (low) to 0.3219 (high) for clinical isolates. (**Figure**

4.2) The data showed that different *S.* Enteritidis strains, including the clinical isolates, exhibited highly diverse virulence levels and different potential to survive in macrophages. When food and clinical strains were compared, the average invasion rate of clinical isolates was about two times that of the food isolates; however, there was no significant difference between the replication rate of the two groups after removal of the abnormally high replication rate of food isolate 860 (**Figure 4.3**).

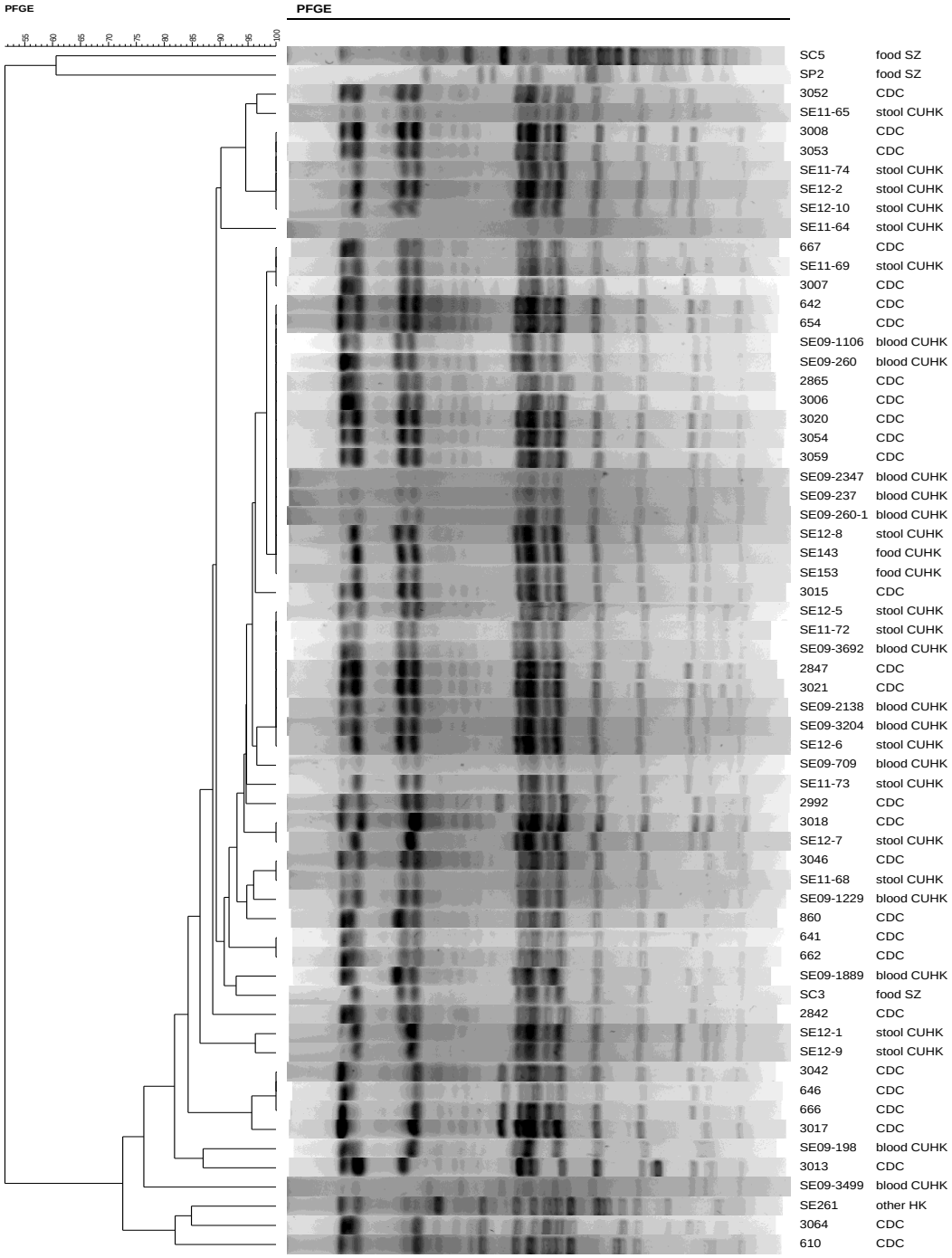

Figure 4.1. Cluster analysis of PFGE profiles of 61 *S.* Enteritidis isolates. Strains labelled as blood CUHK and stool CUHK were isolated from blood or stool samples from patients in Hong Kong. Strains labelled as food SZ were isolated from food samples in Shenzhen, while strains labelled as CDC were isolated from various food samples provided by China CDC.

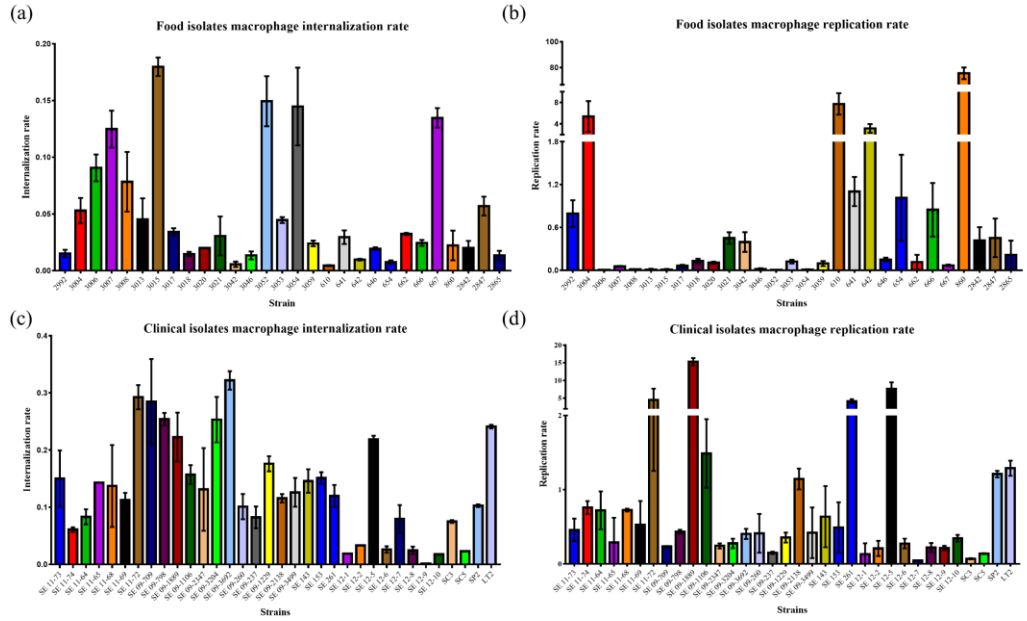

Figure 4.2. Macrophage internalization and replication rate of different S. Enteritidis isolates collected from food and clinical specimens. (a) Internalization rate and (b) Replication rate of food isolates. (c) Internalization rate and (d) Replication rate of clinical isolates. S. Typhimurium LT2 was used as high virulence reference strain.

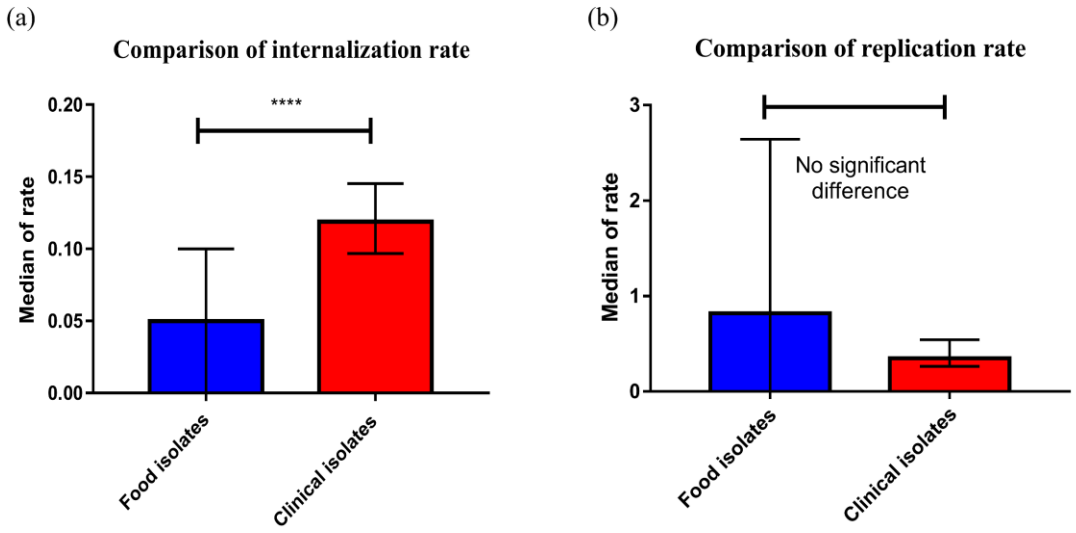

Figure 4.3. Comparison of macrophage (a) internalization rate and (b) replication rate between strains isolated from food and clinical samples.

The 61 *S.* Enteritidis strains were then subjected to screening of 11 known virulence genes to determine if the types and number of virulence genes that each strain harboured contributed to the variation in their ability to invade the human host and survival in

macrophage (**Table 4.2**). Our data showed that the *invA*, *avrA*, *ssaQ*, *sopE1* and *bcfC* genes were present in all 61 isolates; the gene that encodes the Peyer's patch-specific virulence factor, *gipA*, was not detected in all isolates; the other genes were also highly prevalent (an average of over 86%) among the test isolates. Correlation between carriage of specific virulence genes and survival fitness / virulence level of the test strains was not observed, suggesting that variation in virulence level of the test *Salmonella* strains was not entirely due to the presence of specific genetic factors. Correlation analysis of virulence and PFGE profile also showed that there was no difference between the PFGE profiles of isolates of high and low virulence level (**Figure 4.1**). Nevertheless, some clinical isolates shared identical PFGE pattern with foodborne strains. This observation is consistent with the general view that clinical *S.* Enteritidis strains originated from food samples. Twelve *S.* Enteritidis isolates, including six high virulence clinical isolates and six foodborne strains of low virulence level, were then subjected to WGS analysis (**Table 4.3, Table 4.4, Table 4.5**). Comparative genomic analysis confirmed that the genetic compositions of highly virulent isolates were identical to those of low virulence strains (**Table 4.3**).

Up-regulation of virulence, regulatory and metabolic gene clusters in high virulent *S.* Enteritidis strains

In an attempt to delineate the cellular basis of differential virulence levels observed in genetically identical strains, six selected *S.* Enteritidis strains which exhibited different virulence levels were subjected to RNA sequencing and then transcriptome analysis (**Table 4.6**). Among these six strains, three were isolated from food samples (2992, 3046 and 654) and the other three were isolated from clinical samples (SE 12-5, SE 11-72 and SE 09-1889). Based on their macrophage internalization and replication rate, strains 2992 and 3046 were regarded as low virulence organisms, 654 was regarded as

an intermediate virulence strain, and SE 12-5, SE 11-72 and SE 09-1889 were high virulence strains. Gene expression analysis showed that the low virulence strains (2992 and 3046) produced a lower level of mRNA in a wide range of known virulence and host adaptation genes when compared to strain (654) which exhibited intermediate virulence, and the high virulence strains SE 12-5, SE 11-72 and SE 09-1889. It should be noted that the expression level of the virulence genes in intermediate virulence strain 654 was similar to that of the high virulence strains. Based on the mRNA expression and virulence phenotypic data, 2992 and 3046 were confirmed to be low virulence strains, whereas strain 654 was then re-defined as high virulence strain, along with strain SE 12-5, SE 11-72 and SE 09-1889. Consistently, the over-expressed virulence factors of *S.* Enteritidis identified in this work include genes involved in fimbriae synthesis pathway, those located in *Salmonella* Pathogenicity Island (SPI) 1, SPI2, and those of the Type I secretion system, confirming that over-expression of these virulence genes is involved in invasion and survival of *S.* Enteritidis. On the other hand, we discovered that a number of metabolic gene clusters were over-expressed in high virulence strains compared to those in low virulence strains which were previously not regarded as being related to *Salmonella* virulence. These gene clusters include those involved in maltose transport, citrate metabolism, vitamin B12 biosynthesis, propanediol utilization, cytochrome biogenesis, hydrogen production and nitrite reduction. Furthermore, we also identified a number of regulatory genes which were over-expressed in high virulence strain according to results of transcriptome analysis (**Table 4.10**). The expression level of the two-component system *phoQ*, the carbon storage regulator *csrA*, the invasion protein regulator *hilA*, the DNA binding protein Fis, the *araC* family transcriptional regulators *hilC* and *hilD*, the gene encoding the double-stranded RNA ribonuclease RNase III and several other elements with poorly defined functions such as *sicA*, were found to significantly up-regulated in the high virulence group but not the low virulence

strains. This finding indicates that expression of virulence genes may be induced by the upstream regulators which are over-expressed in the high virulence *S*. Enteritidis strains.

Table 4.10. Metabolic pathways and regulatory elements involved in mediating virulence expression in *S.* Enteritidis.

Fold of change*	Genes	Order in Chromosome	Gene function
Regulatory gene			
3.6	-	SEN4082	hypothetical protein
10.2	*gerE*	SEN4085	GerE family regulatory protein
10.2	*araC*	SEN4086	AraC family regulatory protein
3.0	-	SEN4087	hypothetical protein
5.9	*ramA*	SEN0551	transcriptional activator RamA
7.0	*yncC*	SEN1467	DNA-binding transcriptional regulator
6.3	*hilA*	SEN2718	invasion protein regulator
4.7	*yaiV*	SEN0357	DNA-binding transcriptional regulator
4.6	*sprB*	SEN2708	AraC family transcriptional regulator
4.5	*invF*	SEN2740	AraC family regulatory protein
3.8	*stpA*	SEN2643	DNA binding protein
3.3	*hilD*	SEN2717	AraC family transcriptional regulator
3.1	*yhjB*	SEN3429	LuxR family transcriptional regulator
3.1	*hilC*	SEN2709	AraC family transcriptional regulator
2.4	*tdcA*	SEN3086	DNA-binding transcriptional activator TdcA
2.3	-	SEN0709	LysR family transcriptional regulator
2.5	*recO*	SEN2559	DNA repair protein RecO
2.2	*era*	SEN2560	GTP-binding protein Era
2.2	*rnc*	SEN2561	Ribonuclease III
Fimbriae synthesis pathway			
32.9	*fimA*	SEN0524	type-1 fimbrial protein subunit a
29.6	*fimI*	SEN0525	major pilin protein
45.1	*fimC*	SEN0526	fimbrial chaperone protein
25.7	*fimD*	SEN0527	outer membrane usher protein FimD
17.3	*fimH*	SEN0528	FimH protein
13.7	*fimF*	SEN0529	fimbrial protein
21.9	*fimZ*	SEN0530	transcriptional regulator FimZ
4.0	*fimY*	SEN0531	fimbriae Y protein
30.1	-	SEN0532	hypothetical protein

6.7	*fimW*	SEN0533	fimbriae w protein
Salmonella Pathogenicity Island 1			
4.3	*avrA*	SEN2707	pathogenicity island membrane protein
4.6	*sprB*	SEN2708	AraC family transcriptional regulator
3.1	*hilC*	SEN2709	AraC family transcriptional regulator
4.2	-	SEN2710	hypothetical protein
4.4	*orgA*	SEN2711	cell invasion protein
5.2	-	SEN2712	cell invasion protein
4.6	*prgK*	SEN2713	pathogenicity 1 island effector protein
4.4	*prgJ*	SEN2714	pathogenicity 1 island effector protein
4.1	*prgI*	SEN2715	pathogenicity 1 island effector protein
4.3	*prgH*	SEN2716	pathogenicity 1 island effector protein
3.3	*hilD*	SEN2717	AraC family transcriptional regulator
6.3	*hilA*	SEN2718	invasion protein regulator
5.7	*iagB*	SEN2719	cell invasion protein
5.3	*sptP*	SEN2720	tyrosine phosphatase
4.4	*sicP*	SEN2721	chaperone
4.3	*iacP*	SEN2722	acyl carrier protein
4.8	*sipA*	SEN2723	pathogenicity island 1 effector protein
4.3	*sipD*	SEN2724	pathogenicity island 1 effector protein
3.8	*sipC*	SEN2725	pathogenicity island 1 effector protein
4.4	*sipB*	SEN2726	pathogenicity island 1 effector protein
4.0	*sicA*	SEN2727	hypothetical protein
9.2	*spaS*	SEN2728	surface presentation of antigens protein SpaS
5.9	*spaR*	SEN2729	secretory protein
5.3	*spaQ*	SEN2730	secretory protein
6.5	*spaP*	SEN2731	surface presentation of antigens protein SpaP
5.6	*spaO*	SEN2732	surface presentation of antigens protein SpaO
5.9	*invJ*	SEN2733	surface presentation of antigens protein
6.0	*invI*	SEN2734	secretory protein
5.4	*invC*	SEN2735	ATP synthase SpaL
6.7	*invB*	SEN2736	secretory protein
5.4	*invA*	SEN2737	secretory protein
5.3	*invE*	SEN2738	cell invasion protein
5.2	*invG*	SEN2739	secretory protein
4.5	*invF*	SEN2740	AraC family regulatory protein
7.4	*invH*	SEN2741	cell adherance/invasion protein
1.7	*pipA*	SEN0951	pathogenicity island protein

3.6	*pipB*	SEN0952	pathogenicity island protein
5.4	*pipC*	SEN0954	cell invasion protein
4.7	*sopB*	SEN0955	cell invasion protein
3.0	*orfX*	SEN0956	hypothetical protein
2.3	-	SEN0957	hypothetical protein
5.6	*SopE*	SEN1155	type III secretion system, secreted effector protein SopE
4.3	-	SEN1157	hypothetical protein
4.8	*sopD*	SEN2784	hypothetical protein
Salmonella Pathogenicity Island 2			
5.5	*ssaU*	SEN1623	secretion system apparatus protein SsaU
>10	*ssaT*	SEN1624	type III secretion protein
5.2	*ssaS*	SEN1625	type III secretion protein
10.6	*ssaR*	SEN1626	type III secretion system protein
3.4	*ssaQ*	SEN1627	type III secretion system protein
10.8	*ssaP*	SEN1628	type III secretion protein
20.6	*ssaO*	SEN1629	type III secretion protein
3.7	*ssaN*	SEN1630	type III secretion system ATPase
5.0	*ssaV*	SEN1631	secretion system apparatus protein SsaV
10.4	*ssaM*	SEN1632	pathogenicity island protein
11.0	*ssaL*	SEN1633	secretion system protein
7.2	*ssaK*	SEN1634	pathogenicity island protein
11.9	-	SEN1635	pathogenicity island protein
8.5	*ssaJ*	SEN1636	pathogenicity island lipoprotein
7.8	*ssaI*	SEN1637	pathogenicity island protein
7.7	*ssaH*	SEN1638	pathogenicity island protein
4.2	*ssaG*	SEN1639	pathogenicity island protein
1.8	*sseG*	SEN1640	pathogenicity island effector protein
2.6	*sseF*	SEN1641	pathogenicity island effector protein
4.7	*sscB*	SEN1642	pathogenicity island protein
8.6	*sseE*	SEN1643	pathogenicity island effector protein
5.5	*sseD*	SEN1644	pathogenicity island effector protein
4.7	*sseC*	SEN1645	pathogenicity island effector protein
4.2	*sscA*	SEN1646	type III secretion system chaperone protein
4.4	*sseB*	SEN1647	pathogenicity island effector protein
3.1	*sseA*	SEN1648	pathogenicity island protein
4.0	*ssaE*	SEN1649	secretion system protein
14.7	*ssaD*	SEN1650	pathogenicity island protein

4.7	*ssaC*	SEN1651	outer membrane secretory protein
16.6	*ssaB*	SEN1652	pathogenicity island 2 secreted effector protein
3.7	-	SEN2228	exported protein
1.8	*sifA*	SEN1825	secreted effector protein
4.3	*sifB*	SEN1454	secreted effector protein
4.8	*sopD*	SEN2784	hypothetical protein
1.7	*pipA*	SEN0951	pathogenicity island protein
3.6	*pipB*	SEN0952	pathogenicity island protein
5.4	*pipC*	SEN0954	cell invasion protein
4.7	*sopB*	SEN0955	cell invasion protein
3.0	*orfX*	SEN0956	hypothetical protein
2.3	-	SEN0957	hypothetical protein
14.5	*sseJ*	SEN1422	translocated effector protein SseJ
>10	-	SEN1423	hypothetical protein
3.2	-	SEN1423A	hypothetical protein
1.3	*sspH2*	SEN2224	secreted effector protein
2.1	*sseI*	SEN0916	type III secreted protein
Type I secretion system			
18.4	-	SEN4026	hypothetical protein
8.8	-	SEN4027	integral membrane protein
7.7	-	SEN4028	type-I secretion protein
5.6	-	SEN4029	type-I secretion protein
3.0	-	SEN4030	hypothetical protein
9.3	-	SEN4032	type-1 secretion protein
Maltose transporters			
4.4	*malG*	SEN3994	maltose transporter permease
4.5	*malF*	SEN3995	maltose transporter membrane protein
4.4	*malE*	SEN3997	maltose ABC transporter periplasmic protein
7.0	*malK*	SEN3998	maltose/maltodextrin transporter ATP-binding protein
4.5	*lamB*	SEN4000	maltoporin
4.5	*malM*	SEN4001	maltose regulon periplasmic protein
Citrate metabolism			
2.7	-	SEN0054	sensor kinase
4.2	-	SEN0055	oxaloacetate decarboxylase subunit beta
5.0	-	SEN0056	oxaloacetate decarboxylase
3.3	-	SEN0057	oxaloacetate decarboxylase subunit gamma
5.1	-	SEN0058	citrate-sodium symporter
3.5	*citC2*	SEN0059	[citrate (pro-3S)-lyase] ligase

3.4	*citD2*	SEN0060	citrate lyase subunit gamma
3.1	*citE2*	SEN0061	citrate lyase subunit beta
2.7	*citF2*	SEN0062	citrate lyase subunit alpha
2.3	*citX2*	SEN0063	citx protein
2.3	*citG2*	SEN0064	citg protein
B12 biosynthesis and propanediol utilization			
3.1	*pduA*	SEN2036	propanediol utilization protein
3.3	*pudB*	SEN2037	propanediol utilization protein PduB
3.6	*pduC*	SEN2038	glycerol dehydratase large subunit
3.8	*pduD*	SEN2039	diol dehydratase medium subunit
4.4	*pduE*	SEN2040	diol dehydratase small subunit
3.9	*pduG*	SEN2041	propanediol utilization protein
4.5	*pduH*	SEN2042	propanediol utilization protein
3.7	*pduJ*	SEN2043	propanediol utilization protein
3.8	*pduK*	SEN2044	propanediol utilization protein
4.4	*pduL*	SEN2045	propanediol utilization protein
4.5	*pduM*	SEN2046	propanediol utilization protein
4.4	*pduN*	SEN2047	propanediol utilization protein
4.3	*pduO*	SEN2048	propanediol utilization protein
4.5	*pduP*	SEN2049	CoA-dependent proprionaldehyde dehydrogenase
3.7	*pduQ*	SEN2050	propanol dehydrogenase
2.4	*pduS*	SEN2051	propanediol utilization ferredoxin
1.9	*pduT*	SEN2052	propanediol utilization protein
1.7	*pduU*	SEN2053	propanediol utilization protein PduU
1.7	*pduV*	SEN2054	propanediol utilization protein PduV
1.4	*pduW*	SEN2055	propionate kinase
1.7	*pduX*	SEN2056	propanediol utilization protein
Cytochrome biogenesis			
3.2	*ccmH*	SEN2230	cytochrome c-type biogenesis protein H1
3.4	*ccmG*	SEN2231	cytochrome c biogenesis protein CcmG
3.4	-	SEN2232	cytochrome c-type biogenesis protein F1
3.4	*ccmE*	SEN2233	cytochrome c-type biogenesis protein CcmE
5.8	-	SEN2234	heme exporter protein D2
3.2	-	SEN2235	heme exporter protein C2
2.7	-	SEN2236	heme exporter protein B
3.3	-	SEN2237	cytochrome c biogenesis protein CcmA
4.5	*napC*	SEN2238	cytochrome c-type protein NapC
4.9	*napB*	SEN2239	citrate reductase cytochrome c-type subunit

5.0	*napH*	SEN2240	quinol dehydrogenase membrane protein
5.4	*napG*	SEN2241	quinol dehydrogenase periplasmic protein
4.8	*napA*	SEN2242	nitrate reductase catalytic subunit
7.5	*napD*	SEN2243	assembly protein for periplasmic nitrate reductase
4.8	*napF*	SEN2244	ferredoxin-type protein
1.4	*nrfA*	SEN4047	cytochrome c552
1.7	*nrfB*	SEN4048	cytochrome c nitrite reductase pentaheme subunit
1.7	*nrfC*	SEN4049	cytochrome c-type biogenesis protein
2.0	*nrfD*	SEN4050	cytochrome c-type biogenesis protein
2.9	-	SEN4051	heme lyase subunit NrfE
4.2	-	SEN4052	formate-dependent nitrite reductase complex subunit NrfF
2.4	*nrfG*	SEN4053	formate-dependent nitrite reductase complex subunit NrfG
Hydrogen production			
1.6	*hypF*	SEN2683	hydrogenase maturation protein
7.4	*hydN*	SEN2684	electron transport protein HydN
1.0	*hycI*	SEN2687	hydrogenase 3 maturation protease
1.2	*hycH*	SEN2688	formate hydrogenlyase maturation protein
1.4	*hycG*	SEN2689	formate hydrogenlyase subunit 7
1.2	*hycF*	SEN2690	formate hydrogenlyase complex iron-sulfur subunit
1.8	*hycE*	SEN2691	formate hydrogenlyase subunit 5
3.4	*hycD*	SEN2692	formate hydrogenlyase subunit 4
4.5	*hycC*	SEN2693	formate hydrogenlyase subunit 3
7.3	*hycB*	SEN2694	formate hydrogenlyase subunit 2
6.8	*hycA*	SEN2695	formate hydrogenlyase regulatory protein HycA
Nitrite reductase			
6.0	*nirB*	SEN3301	nitrite reductase large subunit
5.1	*nirD*	SEN3302	nitrite reductase small subunit
3.4	*nirC*	SEN3303	nitrite transporter NirC

Metabolic pathways involved in expression of virulence in *S.* Enteritidis

To test the role of metabolic gene clusters in expression of virulence phenotypes in *S.* Enteritidis, the high virulence strain 654 was used as model organism in gene deletion experiments to investigate the effect of gene knockout in virulence expression. Consistently, deletion of fimbriae-encoding operon *fimAICDHF* (*fimAF*) resulted in a decrease in macrophage invasion rate by half (**Figure 4.4a**). Reduction in survival inside macrophages was also observed in Δ*fimAF* (**Figure 4.4b**). Deletion of genes involved in transport and metabolism of various substrates, including the maltose transporter genes *malEFGKM*, the B12 biosynthesis and propanediol utilization proteins-encoding gene *pdu*, the cytochrome biogenesis proteins-encoding genes *nap*, *nrf* and *ccm*, the hydrogen production protein-encoding gene *hyp*, and the nitrite reductase-encoding gene *nir* was also found to result in reduction in macrophage invasion potential (**Figure 4.4a**). In addition, deletion of genes involved in metabolism also resulted in decrease in survival rate of *S.* Enteritidis 654 in macrophages, an exception being the gene encoding the cytochrome biogenesis protein, *nap*, deletion of which resulted in 18-fold increase in the rate of replication inside macrophages (**Figure 4.4b**). The product of *nap* is required for the growth of *Salmonella* under anaerobic condition by enabling the organism to utilize nitrate as electron acceptor during anaerobic respiration[81]. The function of *nap* in catalytic degradation of nitric oxide (NO) produced by the inducible nitrogen oxide synthase (iNOS) in macrophage is not clear. Based on the results of macrophage invasion and survival assay, we observed that deletion of genes in the anaerobic respiratory pathway, including *citCG*, *nrf*, *nir* and *ccm*, results in reduction in virulence level in *Salmonella* (**Figure 4.4**).

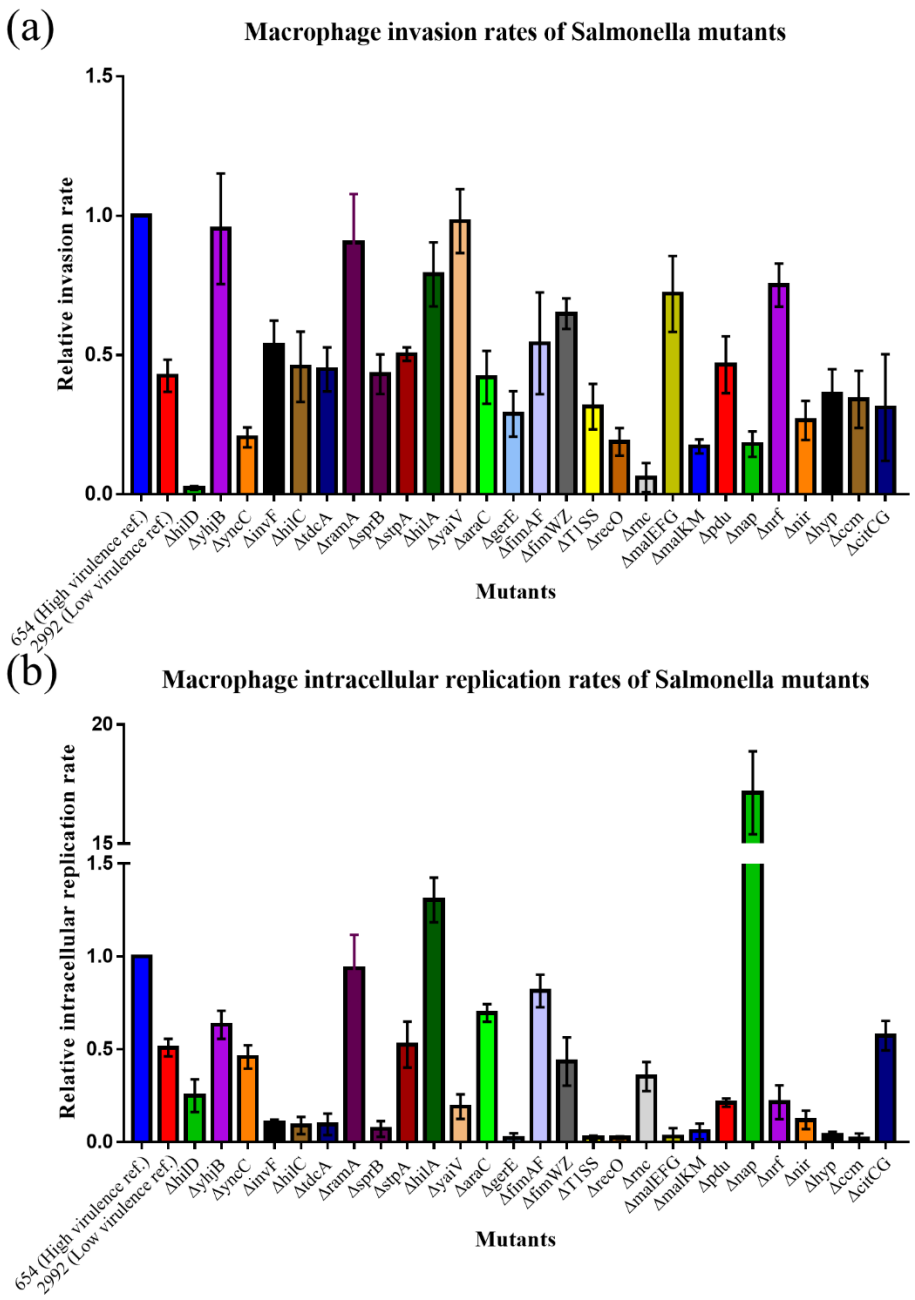

Figure 4.4. Macrophage invasion rate of *Salmonella* knockout mutants. The graph shows the relative rate of invasion (a) and intracellular replication (b) of each *Salmonella* knockout mutant in macrophage RAW264.7. Strain 654 and 2992 were used as high and low virulence reference strains, respectively.

A range of *in vivo* assays were then performed to further test the role of selected genes involved in metabolism in virulence expression in *S*. Enteritidis. The ability of these mutants to colonize the GI tract of mice were tested, with results showing that only the deletion mutant Δ*pdu* exhibited significant reduction in the faecal bacterial count upon inoculation of bacteria (**Figure 4.5a**). The CFU recorded was over 2-$\log_{10}$ less than that of the parental strain 654 from the 3rd day until the 7th day of the experiment, yet the bacterial count returned to the normal level on the 8th day. The gene products encoded by the *pdu* gene cluster are known to play a role in utilizing propanediol for energy production during anaerobic respiration[5]. As conditions in the GI tract of mice were mostly anaerobic, products of the *pdu* cluster may enhance *Salmonella* colonization of the mouse GI tract. GI tract of animals is the habitat of numerous microbes. The infection process of *Salmonella* involves competition with other microbes in the GI tract of the hosts and may be highly dependent on the ability to utilize various compounds in different modes of energy production. Hence, deletion of *pdu* may reduce the virulence level of *Salmonella* through suppression of energy utilization potential.

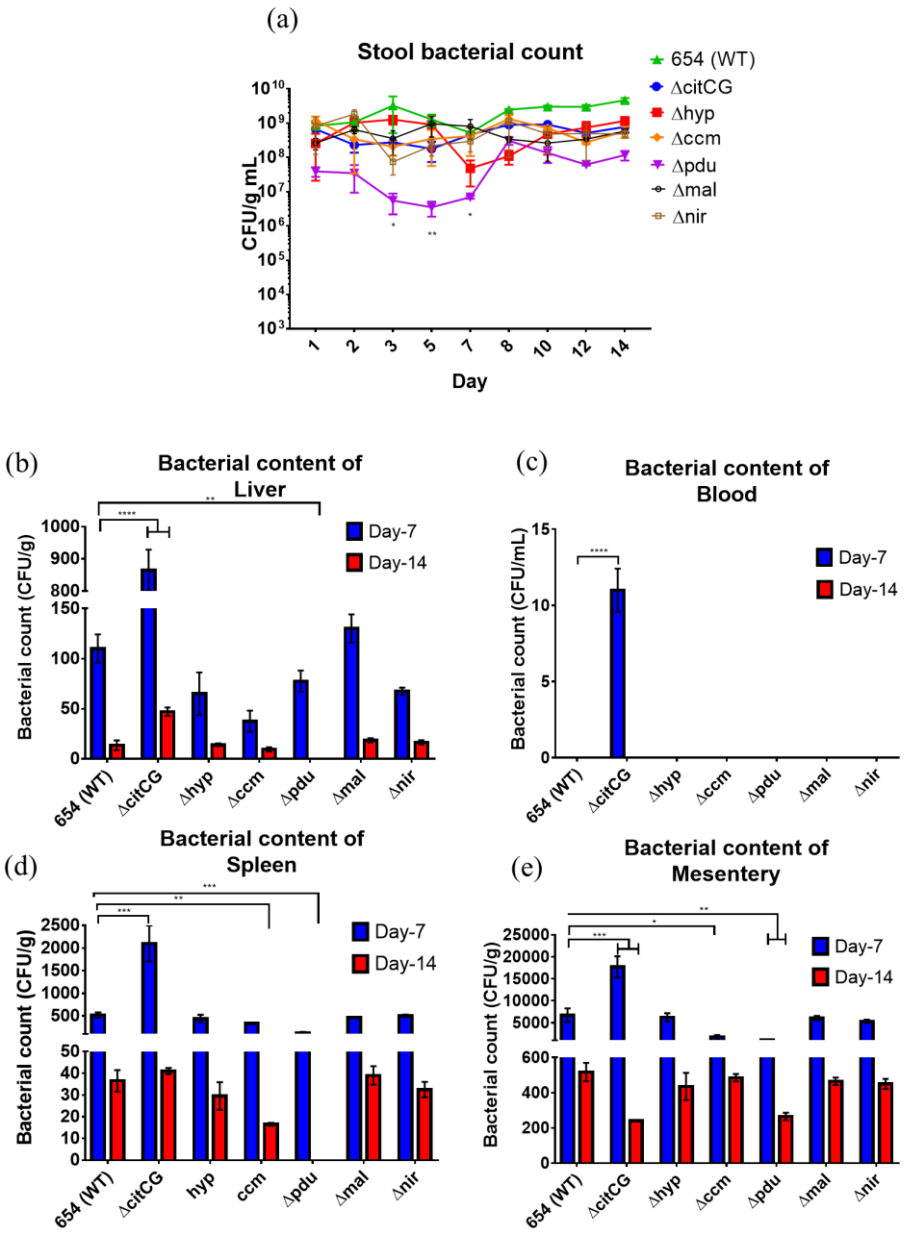

Figure 4.5. In vivo infection assay of non-pathogenic gene cluster knockout mutants. The degree of virulence of strain 654, and the corresponding gene knockout mutants Δ*citCG*, Δ*hyp*, Δ*ccm* Δ*pdu*, Δ*mal*, Δ*nir* were determined using a mouse infection model. Equal amount of vegetative bacterial cells of each strain was introduced into the mice by the oral-feed method. Bacterial load in stool samples collected on Day 14 (a) and blood sample (b), liver tissue (c), spleen tissue (d) and intestinal mesentery tissue (e) collected on Day 7 and Day 14 of the experiment was determined.

Apart from the faecal samples, blood, liver, spleen and intestinal mesentery tissue samples were collected on the 7th and 14th day of experiment. As the intestinal mesentery was the first infection site of invasive infection of *Salmonella*, the CFU count of intestinal mesentery could directly reflect the invasiveness of the test strains (**Figure 4.5e**). Over 5000 CFU/g of *S.* Enteritidis 654 were recorded in intestinal mesentery on the 7th day, but the count dropped to about 500 CFU/g on the 14th day. This change may be due to recovery of intestinal inflammation of the mice when the infection process is put under control by the immune response of the animal. The CFU of the Δ*pdu* mutant, at 1100 CFU/g, was about 1/5 that of the parental strain on the 7th day. This finding, which shows that deletion of the *pdu* cluster results in reduction in the replication rate of *S*. Enteritidis, as well as reduction in invasiveness of *S.* Enteritidis into the intestinal mesentery, supports the idea that this gene cluster is important for *S*. Enteritidis colonization in the GI tract. (**Figure 4.5e**). The CFU count of Δ*pdu* in the liver and spleen was slightly less than that of the wild-type strain on the 7th day and no CFU was recorded in both organs on the 14th day, suggesting that VitB12 biosynthesis and propanediol utilization of *S*. Enteritidis were important for GI tract colonization and further invasion into the extraintestinal sites. This observation further confirms that the *pdu* cluster is an important determinant of virulence in *S*. Enteritidis.

The bacterial load of the Δ*citCG* mutant was about three times that of the parental strain on the 7th day but was about half on the 14th day, indicating that deletion of *citCG* resulted in more efficient invasion into the intestinal epithelium, but such mutant did not survive well in macrophage and were cleared by macrophages residing in lamina propria. Interestingly, the bacterial count in blood was relatively low and were not readily observable by the plate count method, except for mice infected by Δ*citCG*, in which 11 CFU per mL of blood was recorded on day 7th of the infection experiment

(**Figure 4.5c**). The blood CFU data matched that of the intestinal mesentery, showing that Δ*citCG* has higher potential to invade into the blood stream of mice. On the other hand, bacteria were found to be accumulated in liver (**Figure 4.5b**) and spleen (**Figure 4.5d**). As much as 110 CFU/g of *S*. Enteritidis strain 654 could be detected in the liver tissue on day 7th, but the cell count decreased to about 10 CFU/g on the 14th day. The CFU of Δ*citCG* in the liver tissue was about eight folds that of the wild-type strain 654 on the 7th day and about 5 folds on day 14th day of the experiment. A similar trend was also observed in the spleen tissue, in which high CFU count of Δ*citCG* was recorded on day 7th day but the cell count decreased to a level similar to that of wild-type strain 654 on day 14th. The difference in bacterial count recorded in different organs on day 7th of the infection experiment suggests that deletion of the *citCG* cluster results in increase in invasiveness of *S*. Enteritidis. Data on bacterial count on the 14th day of the experiment suggests that the bacterial cells were being cleared by the residential macrophages in liver and spleen at the late stage of the infection experiment. The *citCG* cluster encodes the enzyme citrate lyase, which is a key component in the anaerobic citrate fermentation pathways that catalyse the breakdown of citrate to pyruvate [7]. Deletion of the *citCG* cluster may therefore cause accumulation of excessive citrate. It was reported that an elevated level of citrate could trigger expression of inflammasome complexes in the host, resulting in acute inflammation [132]. Inflammation of the intestine may in turn promote internalization of *Salmonella* into the epithelial cells [108]. The enhanced inflammatory response also promotes clearance of bacterial cells by macrophages.

Reduced invasion into the intestinal mesentery also observed in the Δ*ccm* mutant. The Ccm system was found to be important for utilizing sulphate compounds for energy production via different modes of respiration, including anaerobic respiration[115].

This finding suggests that invasion of *S*. Enteritidis in the intestinal environment was promoted by the sulphate-dependent respiratory pathways. The bacterial count in the mesentery samples of mice infected by the Δ*hyp*, Δ*mal* and Δ*nir* mutants was similar to mice infected by the wild-type strain 654 on both the 7th and 14th day of the experiment. The CFU in other organs also remain unchanged, suggesting that components of these metabolic pathways were not associated with GI tract colonization and invasion into extra-intestinal sites.

Role of regulatory genes in virulence expression of *S.* Enteritidis

Apart from testing the functional role of selected metabolic gene clusters in virulence expression, regulatory genes that exhibited high expression levels in the high virulence *S*. Enteritidis strains were also tested in gene knockout experiments to determine if they played a role in regulating the virulence level of *Salmonella*. As expected, deletion of the *Salmonella* pathogenicity regulator *hilD* abolished the ability to undergo invasion; Deletion of the AraC/XylS family regulator *invF*, which is also known as type-III secretion apparatus required for invasion, resulted in impairment of macrophage invasion to a level comparable to that of the low virulence strain 2992. Deletion of other regulatory genes, including *yhjB*, *yncC*, *hilC*, *tdcA*, *sprB*, *stpA*, *hilA*, *yaiV*, *araC*, *recO* and *gerE*, also resulted in decrease in macrophage invasion potential (**Figure 4.4a**). Deletion of known *Salmonella* virulence regulatory genes such as *hilD* and *invF* resulted in reduction in bacterial survival in macrophage in a manner similar to other the transcriptional regulators *yhjB*, *yncC*, *stpA*, *yaiV*, *araC*, *gerE*, *recO* and *rnc* (**Figure 4.4b**). Among these regulatory genes, two novel regulatory genes that were most likely involved in regulation of virulence expression in *S*. Enteritidis based on results of gene deletion experiments were chosen for further investigation. Deletion mutants of these two genes, Δ*yaiV* and Δ*yhjB*, were tested for their ability to adhere to and invade CaCO-

2, using strain 654 and Δ*hilD* as high virulence and attenuated-virulence control, respectively. Our data showed that no effect on adhesion was observable upon deletion of the *yaiV* gene, whereas the Δ*yhjB* mutant exhibited only one-third of the adhesion strength of the parental strain 654 (**Figure 4.6a**), which was even lower than that of the Δ*hilD* mutant. The invasiveness of the Δ*yhjB* mutant was only half of that of the wild-type *S*. Enteritidis strain, whereas the mutant Δ*yaiV* exhibited slightly higher invasion rate than the wild-type *S*. Enteritidis (**Figure 4.6b**). These data suggest that *yhjB* may play a role in up regulating the genes involved in invasion of GI tract epithelium, whereas *yaiV* may be involved in macrophage survival.

The role of these regulatory genes in mediating expression of virulence genes was also tested in a mouse model. Data collected from the mouse GI tract colonization experiments showed that both Δ*hilD* and Δ*yaiV* exhibited reduction in invasion of the mesentery by one log when compared to strain 654, while Δ*yhjB* exhibited 2-fold reduction in the ability to invade when compared to 654 (**Figure 4.6h**). The data was not consistent with the *in vitro* invasion data, presumably due to the fact that survival and invasion of *S*. Enteritidis in various cell types in the GI tract vary significantly. The presence of S. Enteritidis in different organs was also investigated. Strain 654 and all tested deletion mutants could not be detected in blood (**Figure 4.6e**); the bacterial load of the Δ*hilD* and Δ*yaiV* mutants dropped significantly in spleen and liver, whereas that of Δ*yhjB* was similar to strain 654 in liver and lower in spleen (**Figure 4.6f & h**). In the sepsis model (**Figure 4.6i**), a 100% mortality rate was recorded for strain 654 within 48 hours; mortality rate of infection by Δ*yhjB* was slightly lower, reaching 100% at 84-hour; the rate was 40% at 120 hours for the Δ*yaiV* mutant. In comparison, bloodstream infection due to the Δ*hilD* mutant resulted in 40% mortality at the end of experiment (168-hour). Results obtained from these experiments, which tested the virulence level

of various deletion mutants using a mouse sepsis model, are consistent with results of experiments which tested the GI tract colonization potential of the mutants in that the Δ*yaiV* mutant exhibited lower virulence, the level of which was comparable to the virulence-attenuated mutant Δ*hilD*. The Δ*yhjB* mutant also appeared to exhibit reduced virulence. Our data therefore show that at least two regulatory genes, *yhjB* and *yaiV*, play a role in expression of *S*. Enteritidis virulence via different regulatory pathways.

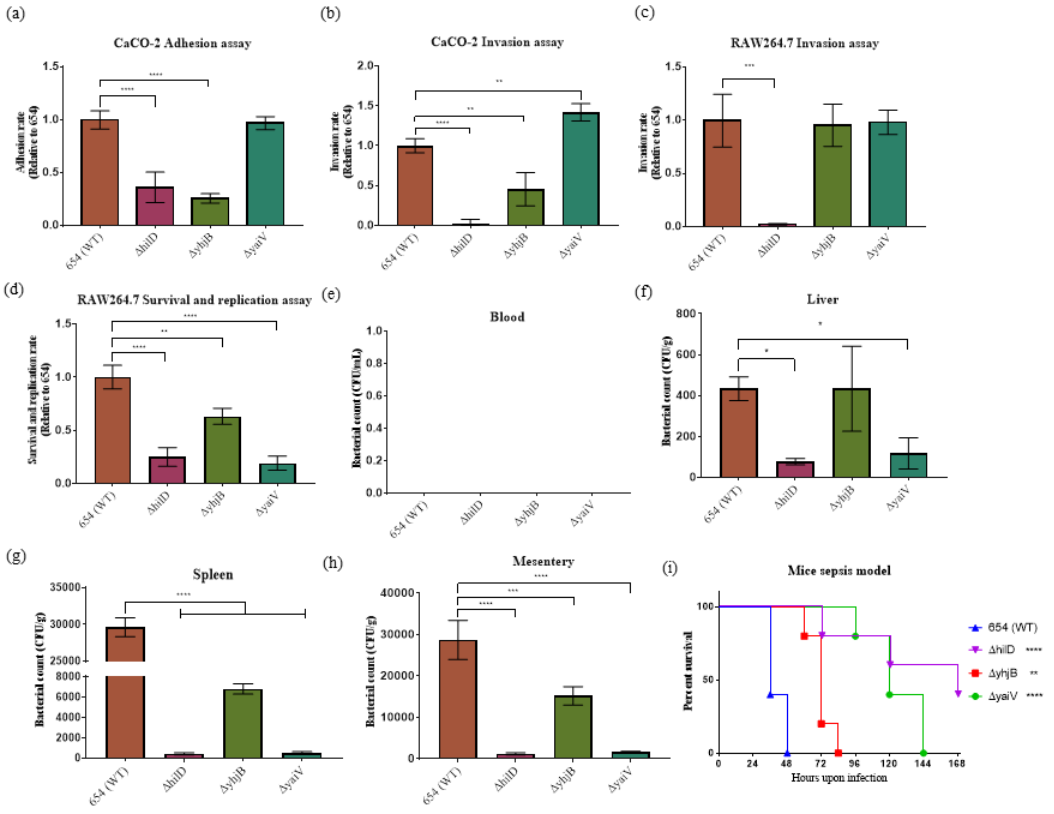

Figure 4.6. Result of gentamicin protection assay and murine infection assay of the Δ*yhjB* and Δ*yaiV S*. Enteritidis strains. The *S*. Enteritidis mutants Δ*yhjB* and Δ*yaiV* were allowed to infect RAW264.7 and CaCO-2 cell line in a MOI=10. **(**a) CaCO-2 cells Adhesion assay (b) CaCO-2 cells invasion assay (c) RAW264.7 invasion assay (d) RAW264.7 survival and replication assay. The murine GI tract colonization model and sepsis model were employed to determine the infection potential of the Δ*yhjB* and Δ*yaiV* mutants in animals. The bacterial load in blood (e), liver (f), spleen (g) and intestinal mesentery (h) were recorded 1 week after oral gavage of *S*. Enteritidis. (i) Survival rate of mice in each experimental group at different time points upon tail vein injection of *S*. Enteritidis suspension.

Delineation of regulatory networks involved in controlling virulence expression in *S*. Enteriditis

The virulence regulatory networks were further investigated by determining the effect of knocking out specific regulatory gene on the expression of other regulatory and virulence genes. Consistent with previous studies, our data also showed that the Δ*hilD* mutant exhibited strongly suppressed expression level of the *invF*, *sprB*, *hilD*, *hilC* and *hilA* genes. We also found that the *invF* and *sprB* gene products positively regulated expression of the *tdcB* gene. Another novel global regulator identified in this study, *yhjB*, was shown to play a similar regulatory role as *hilD*. The knockout mutant Δ*yhjB* was found to exhibit reduced expression level in the regulatory genes which are in turn regulated by *hilD*, *tdcA* and *yaiV*. Deletion of another novel regulator, *yaiV,* also resulted in reduced expression of the *sprB, tdcA* and *hilC* genes. To analyse the effect of deletion of various regulatory genes on the expression of known virulence factors in *S*. Enteritidis, the average expression level of representative genes located in SPI-1, SPI-2, the Fimbriae-encoding genes and the Type I secretion system in specific gene knockout mutants were also tested. Our data showed that deletion of the *invF* gene led to reduced expression of most of the known virulence genes, especially genes in the fimbriae synthesis and Type 1 secretion systems; deletion of the *hilD* gene led to reduced expression of one or more genes in each of these four systems; deletion of *yhjB* resulted in reduced expression in genes in all four systems, except SPI-2. Finally, deletion of *yaiV* mainly led to reduced expression in genes in SPI-2 and the fimbriae synthesis system.

Apart from the transcriptional activators and regulatory proteins, the putative virulence-regulatory role of various metabolic gene clusters, such as those encoding the maltose transporter proteins (*mal*), cytochrome-c biogenesis protein (*ccm*), VitB12 biosynthesis

and propanediol utilization proteins (*pdu*), citrate lyase (*citCG*), nitrite reductase (*nir*) and hydrogen production-related proteins (*hyp*), were also tested by performing gene knockout experiments, followed by qRT-PCR analysis (**Figure 4.7**). The results showed that deletion of the genes encoding the maltose transporter proteins (*mal*), B12 biosynthesis and propanediol utilization proteins (*pdu*), nitrite reductase (*nir*) and cytochrome-c biogenesis protein (*ccm*) resulted in lower expression level of the SPI-1 genes (**Figure 4.7a**). This observation indicates that the *mal* and *nir* genes have virulence-regulatory functions and play a role in mediating transcription of SPI-1. Deletion of the cytochrome-c biogenesis protein-encoding gene *ccm* exhibited similar virulence suppression effect. The known functional role of cytochrome-c proteins in bacteria is to regulate the process of respiration in the electron transport chain[119]. Deletion of *ccm* resulted in reduced efficiency of energy harvesting and affected the expression level of the SPI-1 protein. VitB12 biosynthesis and propanediol utilization functions encoded by *pdu*, and synthesis of the enzyme citrate lyase, encoded by *citCG*, were required for growth of *Salmonella* under anaerobic condition[5, 73]. Deletion of *pdu* gene was found to result in reduction in SPI-1 expression, whereas deletion of *citCG* resulted in gradual increase in expression of SPI-1. Findings in a previous study, which reported that expression of SPI-1 varied in response to changes in the extracellular environment, such as oxygen availability[82], are consistent with results obtained in this work in that metabolites generated from anaerobic respiration may alter the expression level of SPI-1.

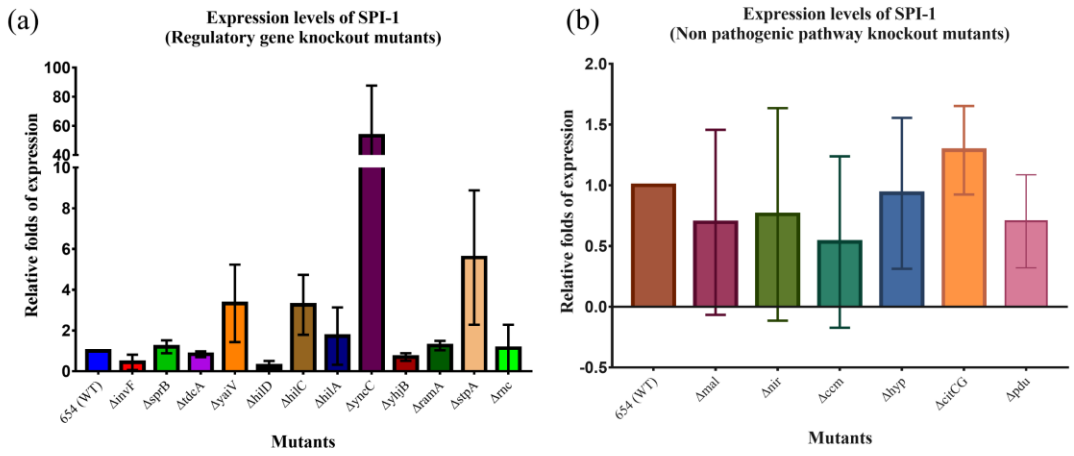

Figure 4.7. Average expression level of SPI-1 genes in (a) regulator gene knockout and (b) non-pathogenic pathway knockout mutants of *S*. Enteritidis.

Discussion

The diversity of virulence phenotypes observable among non-typhoidal *Salmonella* (NTS) strains was believed to be due to serovar variation, and carriage of various chromosomal and plasmid-borne virulence genes[17] [99, 110]. In this study, we adopted a novel approach to delineate novel virulence regulatory mechanisms that could not be discovered using traditional approaches. We speculated that only a small set of *Salmonella* strains harboured by animals and food samples could cause infection in human. Based on this assumption, we tested the virulence levels of *S.* Enteritidis strains collected from animals, food samples and clinical specimens in order to identify strains that exhibited low and high level of virulence for comparative genomic, transcriptomic and phenotypic analyses. Surprisingly, we found that the strains exhibited almost identical genetic compositions regardless of virulence level, suggesting that variation in virulence level among animal/foodborne and clinical *S.* Enteritidis strains are not due to carriage of specific virulence factors by the high virulence strains. However, we observed over-expression of a wide range of genes that were previously not implicated in virulence expression in the high virulence strains but not the low virulence strains. Among them are several novel regulatory genes and various genes involved in metabolism, such as the maltose transporter genes (*mal*) which code for different subunits of the maltose transporter protein. The maltose transporter is a periplasmic ATP-binding maltose transporting protein which is essential for active uptake of maltose in *Salmonella spp*[6]. Over-expression of the maltose transporter may enhance energy production and hence the chance of survival in the host GI tract. Apart from the maltose transporter, genes that were over-expressed in high virulence strains only included those which encoded various physiological functions, namely citrate metabolism, B12 biosynthesis, propanediol utilization and hydrogen production. These functions are important for maintaining the ability to utilize various

organic compounds for energy production under anaerobic conditions[5, 7, 19]. The nutrient scavenging activity is essential for survival of *S*. Enteritidis under anaerobic condition and is therefore also important for the pathogen to colonize the host GI tract where oxygen is not available to act as the final electron accepter in the electron transport chain. A previous study reported that the cytochrome *c* biogenesis system consisted of four components, which played an important role in redox control of various ions and organic molecules[72]. Although the exact function of cytochrome biogenesis systems in *S*. Enteritidis remains unclear, it is believed that the system is essential for undergoing different modes of respiration[115]. These findings suggest that the virulence level of *S*. Enteritidis may not be determined by the mere existence of pathogenicity islands or other known virulence genes, but also the metabolic status of the organism that controls the expression level of the virulence factors, as well as the ability to utilize various nutrients to generate energy. It should be noted that the metabolic status also determines the survival fitness of the organism in the host and hence indirectly determines the virulence level. These findings therefore suggest that the ability of *S*. Enteritidis to adapt to the host environment is one key factor that determines its virulence level.

Apart from the metabolic gene clusters, we also identified several regulatory genes which play a role in expression of high virulence phenotypes. Among these regulatory genes, several (*invF, sprB, hilD, hilC* and *hilA*) are already known as regulators of SPIs, further indicating that our approach can successfully identify novel virulence regulatory mechanisms in *Salmonella*. Importantly, several regulatory genes, namely *tdcA*, *yaiV*, *yncC*, *yhjB*, *ramA*, *stpA* and *rnc*, which are previously not implicated in virulence expression, were found to play a role in regulating the level of virulence in *Salmonella* based on analysis of gene deletion mutants both *in vitro* and *in vivo*. It was reported that

mutations in the *tdcA* gene would attenuate virulence in *S.* Typhimurium due to impaired degradation of L-serine and L-threonine, which would in turn result in reduced expression of SPI-2[77]. These findings therefore further confirm that the metabolic status of *Salmonella* may affect expression of virulence genes. The *stpA*, *yncC* and *rnc* gene products are involved in the gene transcriptional processes, hence mutations in these genes may affect gene expression, including the virulence genes[2, 4, 83]. However, the functions of *yaiV* and *yhjB* are not clear, except that one previous study reported that the *yaiV* gene was associated with survival of *S.* Typhimurium in macrophages[56]. Our data showed that *yhjB* is an important global regulator with a role similar to that of *hilD*, which acts as a super-regulator that controls multiple secondary regulatory genes and hence the downstream virulence genes in SPI-I, Type-I secretion system and the fimbriae synthesis cluster. Existence of this complex regulatory network has been confirmed in this study both *in vitro* and *in vivo*; for example, the Δ*yhjB* mutant was found to exhibit decreased invasiveness, reduced survival inside macrophages, reduced invasion to different organs, and cause lower mortality in mouse infection model, although the degree of reduction in virulence was lower than that observed for the Δ*hilD* mutant. The function of this important master regulatory gene warrants further investigation.

Unlike Δ*yhjB*, deletion of *yaiV* seemed to have no effect on invasion of CaCO-2 cells *Salmonella*; instead Δ*yaiV* exhibited dramatic reduction in survival in macrophages. Data regarding infection potential, bacterial load in different organs and mortality rate of the test animals for the Δ*yaiV* mutant are similar to those of Δ*hilD.* More specifically, the CFU count of Δ*yaiV* in different organs was comparable with the immunodeficient mutant Δ*hilD*, and the mortality rate recorded for the Δ*yaiV* mutant in animal experiments was significantly lower than that recorded for the wild-type strain,

confirming that the *yaiV* gene was important for survival of *S*. Enteritidis against the host immune response. The finding is also consistent with a previous report which showed that the *yaiV* gene product was involved in oxidative stress resistance and played a role in enhancing survival fitness in the phagosome of phagocytic cells[56]. In the *yaiV* mutant, reduced expression of *sprB*, *hilC* and *yncC*, as well as genes involved in SPI-I, SPI-II and fimbriae synthesis, was observed. The role of *yaiV* in regulating expression of virulence genes may be mediated through the *sprB* and *hilC* regulators located in SPI-1, which are also important for promoting expression of T3SS[91, 105].

Conclusion

We adopted a unique approach to study bacterial pathogenesis and found that a wide range of genes that encode regulatory and metabolic functions play an important role in mediating expression of virulence phenotypes in *Salmonella*. Among these genes are two important global regulatory genes, namely *yhjB* and *yaiV*, which play a role in regulating multiple secondary regulator genes that in turn control expression of virulence in *S*. Enteritidis. This study therefore provides novel insight into how *Salmonella* virulence is regulated at the transcriptional level. Further studies are required to investigate mechanistic details regarding induction of key virulence regulatory genes in the high virulence *Salmonella* strains in different environmental niches, and why such regulators are less actively expressed in the low virulence strains despite the fact that they are genetically identical.

CHAPTER V-High Level Expression of Ribonuclease III in *Salmonella* Enteritidis Enhances Survival Against Host Immune Responses

Abstract

Salmonella is an important foodborne pathogen which comprises strains that exhibit varied virulence phenotypes and the capability of causing invasive human infection. In this study, the gene expression profile of foodborne and clinical *Salmonella* strains that exhibit high- and low-level virulence was investigated, with results showing that the expression level of a number of genes, including the *rnc* gene which encodes the RNase III ribonuclease, were exceptionally high in the high virulence strains. Investigation of the role of *rnc* in mediating expression of virulence phenotypes in *Salmonella* showed that the product of this gene could enhance expression of the superoxide dismutase SodA, which is an essential determinant of survival fitness of *Salmonella* under the oxidative stress elicited by the host immunity. On the order hand, we also discovered that the double stranded RNA (dsRNA) released from *Salmonella* could trigger immune response of the host, and that the high-level expression of the *rnc* gene enabled *Salmonella* to evade the host immunity by reducing the amount of dsRNA accumulated in the bacterial cell. These findings facilitate development of novel antimicrobial treatments through suppression of virulence expression and survival fitness of this important pathogen.

Introduction

Salmonella enterica is one of the most important foodborne pathogens, causing over one million cases of infection in the United States annually[10]. Non-typhoidal *Salmonella* account for the majority of infections. To date, about 2500 serovars of *S. enterica* have been recognized, two of which, namely Typhimurium and Enteritidis, are responsible for most cases of non-typhoidal Salmonellosis[84]. Non-typhoidal *Salmonella* infections are mostly associated with self-limiting diarrhoea. However, invasion of the pathogen into normally sterile sites, including blood stream and meninges, is possible, resulting in focal infections[113]. Invasiveness of non-typhoidal *S. enterica* into various body sites has been observed worldwide, however, the mechanisms underlying this invasion process remain poorly understood.

In the previous chapter, we observed that *Salmonella* strains collected from food and clinical samples exhibited a highly diverse range of virulence level. When subjected to comprehensive genetic analysis and different virulotyping assays to investigate the relationships between the genetic profiles of the test strains and their virulence level, we found that genetically identical strains might exhibit highly different virulence levels, and that the molecular basis of such difference was due to variation in expression levels of specific gene clusters. Among these over-expressed gene clusters, we found that the *rnc-era-recO* operon was up-regulated in the highly virulent *Salmonella* isolates. The *rnc-era-recO* operon has been identified in various types of bacteria[98]. Among the genes in this operon, the *rnc* gene encodes the RNase III ribonuclease, which specifically cleaves the 5' phosphoryl and 3' hydroxyl ends of double stranded RNA (dsRNA), resulting in formation of a two-nucleotide 3' overhang at each end [3]. It was reported that the *rnc* gene in *E. coli* played an important role in regulation of

protein synthesis[117]. However, the exact functional role of the *rnc* gene product in *Salmonella* is not known.

In this study, we investigated the role of the *rnc* gene in mediating expression of the virulence phenotype in *Salmonella* and its function in regulating gene expression and mediating enhanced resistance to the host immune response. We found that the *rnc* gene is the key determinant of virulence in *Salmonella*. Findings in this work shall facilitate development of novel strategies to suppress the virulence level of *Salmonella*.

Materials and methods

RNA extraction, qRT-PCR and Northern Blotting

The overnight culture of the test strains was first re-inoculated into fresh LB broth and allowed to grow at 37℃ with shaking, until optical density reached 0.5; 1mL of log-phase culture was then treated with the QIAGEN RNAprotect Bacterial Reagent. Total RNA was extracted by the Qiagen RNeasy Bacteria Minikit, followed by DNase treatment. For RNA extraction of RAW264.7 and CaCO-2 cells, the cultured cells were treated with the QIAGEN RNAprotect Cell Reagent, followed by extraction of RNA by the Qiagen RNeasy Cell Minikit. The quality and quantity of RNA was determined by using the Nanodrop spectrophotometer. One μg of total RNA was subjected to reverse transcription using Life technologies Superscript III reverse-transcriptase. Real-time RT-PCR was performed by using the Applied Biosystem Quant Studio 3 and the Life Technologies SYBR Select Master mix. Melt curve analysis of PCR product was performed to ensure specificity of the selected primers. Expression levels of the test genes were normalized with a housekeeping gene that encodes the DNA gyrase subunit B for bacterial samples or GAPDH protein for cell samples. Primers used in qPCR are listed in **Table 5.1**.

For the Northern Blot assay, bacterial total RNA was quantified and an equal amount of RNA was separated on agarose gel, followed by transfer to PVDM membrane and detection using J2 antibody. The specificity of J2 antibody against double-stranded RNA was confirmed using commercial dsRNA (New England BioLabs #N0363S) and ssRNA (Thermo Scientific #SM1821) ladders.

1 **Table 5.1. Primers used in this study.**

Description	Primer name	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Primers for construction of *rnc* knockout mutant	*rnc*-FRT-F/ *rnc*-FRT-R	ATGAACCCCATCGTAATTAATCGGCTTCAAC GGAAGCTGGGCTACACTTTATGGGAATTAG CCATGGTCC	TCATTCCAACTCCAGTTTTTTCAGCGCCTGTT CGGCGGCAGCCTGCTCCGGTGTAGGCTGGAG CTGCTTC
Primers for construction of *sodA* knockout mutant	*sodA*-FRT-F/ *sodA*-FRT-R	ATGAGTTATACACTGCCATCCCTGCCGTACG CTTATGATGCACTGGAACCATGGGAATTAGC CATGGTCC	TTATTTTTTAGCGGCGAAACGCGCTGCTGCTT CGTCCCAGTTCACCACGTGTGTAGGCTGGAG CTGCTTC
Primers for cloning the *rnc* gene	*rnc*-NdeI/*rnc*-XhoI	CGATCATATGCACACGAAACAGCGTTGGTT	CGATCTCGAGTCATTCCAACTCCAGTTTTTTC AG
Primers for cloning the *sodA* gene	*sodA*-BamHI/*sodA*-XhoI	CGATGGATCCCCCTGGAAAAAGTACGGCAT	CGATCTCGAGTTATTTTTTAGCGGCGAAACG
Primers for assessing expression of the *rnc* gene by qPCR	*rnc*-qF/*rnc*-qR	AAAAAGCGGCGGATTCCGTCG	GGATTAACTGCTCGACGGTCTGG
Primers for assessing expression of the *sodA* gene by qPCR	*sodA*-qF/*sodA*-qR	CGGCTATCGAGCGTGACTTC	CAGTTTGTCGCCTTTCAGCA
Primers for assessing expression of the *RIG-I* (RAW264.7) gene by qPCR	m*RIG-I*-qF/ m*RIG-I*-qR	ACACTAAAGGGAGAATGGCAGG	TCCGCTCCATCATCCTCATCA
Primers for assessing expression of the *RIG-I* (CaCO-2) gene by qPCR	h*RIG-I*-qF/ h*RIG-I*-qR	ATGAAGCCATTGAAAGTTGGG	CACCTGCCATCATCCCCTT
Primers for assessing expression of the *MDA-5* (RAW264.7) gene by	m*MDA-5*-qF/ m*MDA-5*-qR	TGCCCAGAAGACAACACAGAC	TATGTCGTCTACGTTCCAGGC

qPCR			
Primers for assessing expression of the *MDA-5* (CaCO-2) gene by qPCR	h*MDA-5*-qF/ h*MDA-5*-qR	AGGAGCAGATTCAGAGGACAG	AACGATGGAGAGGGCAAGTC
Primers for assessing expression of the *IFN-β* (RAW264.7) gene by qPCR	m*IFN-β*-qF/ m*IFN-β*-qR	GCTCCAAGAAAGGACGAACA	TCTTGGATGGCAAAGGCAGT
Primers for assessing expression of the *IFN-β* (CaCO-2) gene by qPCR	h*IFN-β*-qF/ h*IFN-β*-qR	CTAGCACTGGCTGGAATGAGA	TCCTTGGCCTTCAGGTAATGC
Primers for assessing expression of the *GAPDH* (RAW264.7) gene by qPCR	mGAPDH-qF/mGAPDH-qR	TGCACCACCAACTGCTTAGC	GGATGCAGGGATGATGTTCTGG
Primers for assessing expression of the *GAPDH* (CaCO-2) gene by qPCR	hGAPDH-qF/hGAPDH-qR	ACAGTCAGCCGCATCTTCTT	ATCCGTTGACTCCGACCTTC

Macrophage invasion and survival assay

The virulence level of the tested *Salmonella* strains was determined by infecting RAW 264.7 cells and measuring the internalization and replication rate. The bacterial strains were inoculated into fresh LB broth and incubated at 37℃ with shaking, until the optical density of the bacterial cultures reached 0.5. The bacterial cells were harvested by centrifugation and washed once with phosphate buffered saline (PBS). The washed bacterial cells were then resuspended in DMEM cell culture medium, followed by addition to RAW 264.7 (ATCC® TIB-71™) cells pre-coated in a 24-well cell culture plate with a multiplicity of infection (MOI) ratio of 10:1. The plates were then centrifuged at 500 rpm for 5 min to synchronize the infection, followed by incubation at 37C, 5% CO2 for 25 mins. The medium was then removed and replaced by DMEM supplemented with 200 μg/ml gentamicin and subjected to incubation for 1.5 h; DMEM containing 10 μg/ml of gentamicin was then used for the rest of the experiment. The supernatant was removed at 2 and 16 h after infection, the cells were then washed twice with pre-warmed PBS and lysed with 0.2% Triton X-100. Serial dilutions of the lysates (10^0, 10^{-1},10^{-2},10^{-3},10^{-4}) were plated onto LB agar to enumerate the intracellular bacteria.

ROS assay

Overnight cultures of the test *Salmonella* strains were diluted in LB broth at OD600 of 0.08 and incubated at 37 °C with aeration until OD600 reached 0.3, followed by addition of 10mM H_2O_2. 1 ml culture aliquots were collected at 2, 3.5, 5 and 6.5 hour upon the addition of H_2O_2. The bacterial cells were pelleted and washed three times with phosphate-buffered saline (PBS), and resuspended in 500 μl of PBS containing 5 μM CM-H2DCFDA after the final wash, followed by incubation at 37°C in dark for 30 min. The labelled cells were washed once with PBS and resuspended in 500 μl of PBS. The fluorescence signal of a 200 μl aliquot was measured by Molecular Devices

SpectraMax iD3 at 485±5nm excitation and 535±5nm emission wavelengths. The results were normalized according to the viable cell counts. Briefly, 100µl were removed and subjected to 10-fold dilution. 100µl from various dilutions were spread onto LB agar and incubated at 37 °C until colony forming units (CFUs) formed. CFU was counted in the various dilutions which produced distinct and countable colonies (25-250 CFUs).

Gene knockout and cloning experiments

Gene knockout mutants of *Salmonella Enteritidis* strain 654 were generated by the λ-red system, following the protocol described by Ruth, et al[109]. The pKD46 plasmid was used as the helper plasmid and pKD4 plasmid was used as the template of kanamycin resistance gene. The mutants produced and the sequence of primers used are listed in **Table 5.1**. PCR was performed by using the high-fidelity polymerase to ensure the integrity of gene sequences. The voltage of electroporation was 18kV/cm. The mutants were recovered by addition of pre-warmed SOC medium and incubated at 37℃ with shaking for 2 hours. The recovered cells were selected on agar plates supplemented with kanamycin (50µg/mL). Deletion of the target gene was confirmed by Sanger sequencing.

Gene cloning was performed by using the double restriction digestion method. Briefly, the pET-28b plasmid was chosen as the vector of desired gene sequences. The desired genes were amplified by high-fidelity PCR. Both the vector and the PCR product were digested by restriction enzymes at 37℃ for 4 hours, followed by ligation at 16℃ overnight, using T4 ligase. The ligation products were transformed into *E. coli* strain DH5α, followed by selection of transformants which had acquired the cloned gene. The genetic sequence of the selected clones was confirmed by Sanger sequencing. The

recombinant plasmid harboured by the selected clones was then extracted and transformed into the target strains. The sequence of primers used are listed in **Table 5.1**.

Murine sepsis infection model

ICR mice aged 5 weeks were used as the host for *S.* Enteritidis infection. Each experimental group consisting of 5 mice was infected by different mutants of *S.* Enteritidis. Briefly, *S.* Enteritidis mutants were first grown in LB broth until the optical density at 600nm reached 0.5. The bacterial cells were harvested and washed once with sterile 0.9% sodium chloride solution. The washed bacterial cells were then inoculated into 0.9% sodium chloride solution. The bacterial suspensions were injected into the mice through tail vein at the final dosage of 10^5 bacterial cells. Water and food were given to each mouse during the experiment. The death rate of the mice in each experimental group was recorded at 12-hour intervals.

Western blotting of SodA

Salmonella strains which harboured the desire constructs were first streaked on LB agar plate to ensure no contamination of the stock. The single colonies of each test strain was inoculated into LB broth and incubated overnight at 37℃ with shaking. The culture was re-inoculated to fresh LB broth and incubated at 37℃ with shaking until the absorbance at 600nm reached 0.5. The bacterial cells in 1 ml culture were pelleted by centrifugation and resuspended in 400μL SDS loading buffer, then boiled for 10 minutes. Solubilized proteins were separated by SDS-PAGE and subsequently transferred to the PVDF membrane through Bio-Rad Trans-Blot Turbo Transfer System. Western blotting was carried out by probing the membrane with rabbit anti-SodA polyclonal antibody, followed by goat anti-rabbit IgG. The signal was developed by the addition of HRP-substrate and visualized by Bio-Rad ChemiDoc Imaging System. *Salmonella* GAPDH-specific antibody was used as endogenous loading control.

Transfection of RNA

The corresponding cell line was cultured in a six-wells plate, with cell density of 10^6 cells per well. A total of 2.5 μg RNA was used for the transfection process of each well. Briefly, total RNA extracted from *Salmonella* strains carrying the constructs was first diluted with the Opti-MEM reagent (Gibco™ #A4124801) and mixed with the Lipofectamine 2000 transfection reagent (Invitrogen™ # 11668019) to form RNA-lipid complexes. The complexes were then added to active cell cultured in DMEM. The transfection process was allowed to proceed overnight at 37℃ under a supply of 5% CO_2.

Statistical analysis

All data were presented as the mean ± SD. One-way ANOVA analysis of variance was used to calculate the differences between various experimental groups. A two-tailed value of $P < 0.033$ was regarded as statistically significant. *$P < 0.033$, **$P < 0.002$, ***$P < 0.0002$, ****$P<0.0001$.

Results

The rnc gene was over-expressed in high virulence strains

A total six *S.* Enteritidis strains were recovered from food and clinical samples. Their virulence phenotypes were characterized by determining the internalization and intracellular replication rate of these strains in macrophage RAW264.7 (**Table 5.2**). The macrophage internalization and intracellular replication rate of the food isolates (654, 2992 and 3046) ranged from 0.0075 to 0.0152, and 0.0370 to 1.0150 respectively. Those of the clinical isolates (SE 12-5, SE 11-72 and SE 09-1889) ranged from 0.2189 to 0.2925, and 4.4744 to 15.3199 respectively. The results indicated that the food isolates generally exhibited a lower level of virulence when compared to the clinical isolates. However, it should be noted that strain 654 exhibited exceptionally high virulence among the three food isolates tested, suggesting that the high virulence *Salmonella* strains exist in the natural environment. All test strains were further subjected to RNA sequencing, with results showing that specific genes were over-expressed in the high virulence isolates recovered from the clinical samples as well as the food isolate 654. These highly expressed genes include a number of virulence determinants (e.g. Type-3 secretion system and fimbriae protein-encoding genes), transcription regulators (e.g. *araC* family transcription regulators and the *rnc-era-recO* operon) and various genes that encode cellular functions related to metabolism. Among these genetic elements, the *rnc* gene which encodes RNaseIII was found to be over-expressed in all high virulence *S.* Enteritidis strains (**Figure 5.1a**).

As the function of *rnc* gene product RNase III ribonuclease involves degradation of dsRNA, it appears that alteration of virulence level in *Salmonella* is due to intracellular accumulation of dsRNA. In order to test whether dsRNA was accumulated in the *rnc* knockout mutant, Northern Blot assay was performed. The *Salmonella* strains, SEBL,

SEST, SEBLΔ*rnc* and SESTΔ*rnc* were being tested in this experiment. Upon being cultured for 3 hours and 12 hours, total RNAs were extracted from them and the quantity of dsRNA in each sample was determined using J2 antibody (**Figure 5.1b**). The results showed that dsRNA was not detectable in both 3- and 12-hours culture in the SEBL and SEST strains. However, dsRNA could be detected in the 3 hours culture of the SEBLΔ*rnc* and SESTΔ*rnc* strains; the quantity of dsRNA was even higher in the 12 hours culture. This finding indicates that the gene product of *rnc*, RNase III, was responsible for the degradation of dsRNA in *Salmonella* and that low level of dsRNA correlates with high level virulence in *Salmonella*.

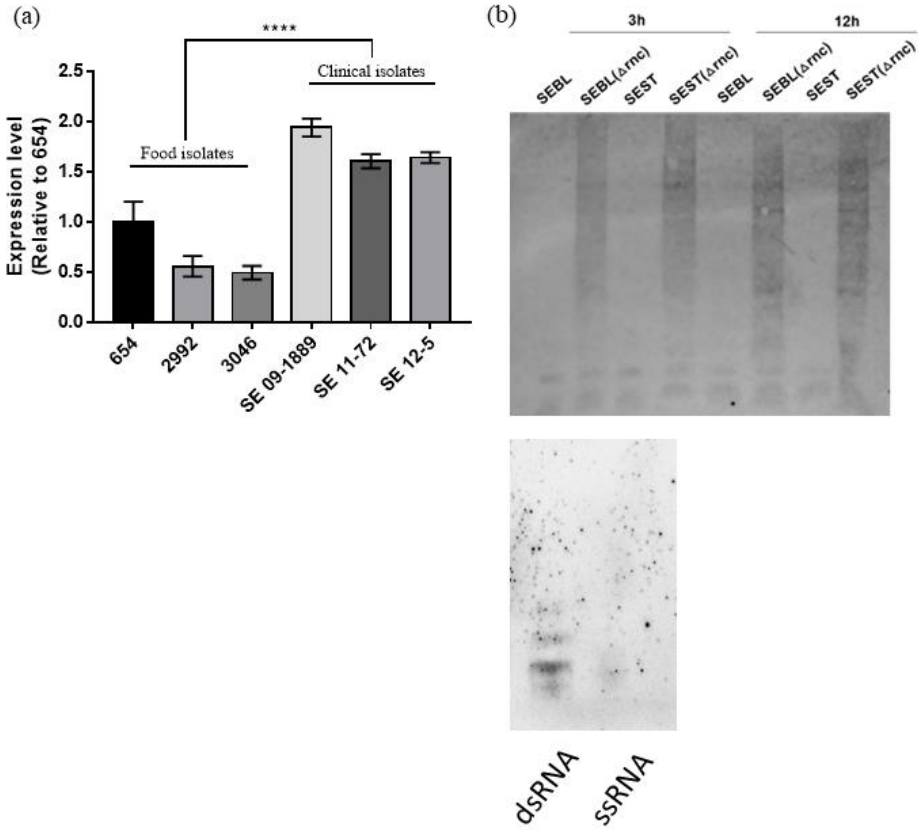

Figure 5.1. RNase III was over-expressed in high virulence strains and was responsible for degradation of dsRNA. (a) Expression level of the *rnc* gene in six *S.* Enteritidis isolates. (b)Two clinical isolates (SEBL, SEST), and their corresponding *rnc* knockout mutants (SEBLΔ*rnc*, SESTΔ*rnc*) were cultured for 3h and 12h. Their total RNAs were extracted, and the dsRNA was detected using J2 antibody. Same amount of total RNA from each sample were loaded. The specificity of J2 antibody on dsRNA was checked by using commercial dsRNA and ssRNA ladder.

Observation of high intracellular replication rate of the test isolates (**Table 5.2**) was highly consistent with high expression level of the *rnc* gene. These findings suggest that the *rnc* gene may play a role in mediating expression of phenotypic virulence in *S.* Enteritidis. It should be noted that sequencing studies cannot reveal the genetic basis of the significant difference between the expression level of the *rnc* gene in the high and low virulence strains at this stage.

Table 5.2 The macrophage internalization and replication rate of six S. Enteritidis strains.

Strains	Source	Location	Year	Internalization rate	Replication rate
2992	food	China CDC	2012	0.0152	0.7934
3046	food	China CDC	2012	0.0135	0.0370
654	food	China CDC	2012	0.0075	1.0150
SE 12-5	stool	CUHK	2012	0.2189	7.6038
SE 11-72	stool	CUHK	2011	0.2925	4.4744
SE 09-1889	blood	CUHK	2009	0.2228	15.3199

The rnc gene was essential for expression of virulence phenotype in Salmonella

To further confirm the role of the *rnc* gene product in regulation of virulence expression in *Salmonella*, the *rnc* gene was deleted from the high virulence foodborne isolate 654 to create the 654Δ*rnc* mutant, which was then subjected to internalization and replication assay in macrophage RAW264.7 (**Figure 5.2a**). The result showed that both the macrophage internalization and intracellular replication rate of *S.* Enteritidis 654 were reduced by up to 80% when the *rnc* gene was deleted from the genome. Consistently, the internalization and intracellular replication rate of the 654Δ*rnc*/p-*rnc* strain, which was created by transformation of a *rnc* gene-bearing plasmid into 654Δ*rnc* mutant to complement the lack of *rnc* in the mutant, were found to have reverted back to the same level as *S.* Enteritidis 654. This finding confirms that the *rnc* gene was important for survival of *Salmonella* in the macrophage and hence the overall infection potential of this important pathogen. In addition, murine sepsis infection assay was

performed to investigate the effect of *rnc* on the ability of *S*. Enteritidis 654 to withstand the host immune response (**Figure 5.2b**). In the experimental group of mice which were injected with *S*. Enteritidis 654, 100% death rate was recorded 84 hours post-infection. On the other hand, mortality of mice injected with 654Δ*rnc* was only 50% 132 hours post-infection, increasing to 100% 144 hours post-infection. For the experimental group injected with the 654Δ*rnc*/p-*rnc* strain, mortality reached 100% 60 hours post-infection, which was comparable to that of the parental strain 654. The results of both in *vitro* and in *vivo* assays indicate that the *rnc* gene is a key virulence determinant of *Salmonella*.

The rnc gene regulates expression of the superoxide dismutase SodA

The results of macrophage infection assays indicated that intracellular survival and replication of *S*. Enteritidis 654 in macrophages were regulated by the *rnc* gene product. Since the survival of *Salmonella* in macrophage was reported to be highly dependent on its ability of reactive oxygen species (ROS) detoxification, we further tested the ROS level in the six *Salmonella* isolates upon the treatment with hydrogen peroxide (**Figure 5.2c**)[100]. As expected, significantly lower level of ROS was detectable in the high virulence strains SE09-1889, SE11-72 and SE12-5 when compared to the low virulence strains 2992 and 3046, whereas intermediate level of ROS was recorded in strain 654. Results of this experiment therefore showed that the ROS level in *Salmonella* was inversely proportional to their virulence level as well as the expression level of *rnc* (**Figure 5.1**). Based on these observations, we hypothesize that *rnc* may up-regulate expression of superoxide dismutase in *Salmonella* and that this is one of the reasons why *rnc* is important for survival of *Salmonella* against the host immune response. To test this hypothesis, qPCR was performed to investigate the expression of the *sodA* gene in strain 654Δ*rnc* (**Figure 5.2e**). However, the level of mRNA transcript of *sodA* in this mutant strain was found to be much higher than that of its parental strain *S*. Enteritidis

654. On the other hand, the level of mRNA transcript of *sodA* in 654Δ*rnc*/p-*rnc* was similar to that of strain *S*. Enteritidis 654. These findings therefore showed that the absence of the *rnc* gene actually resulted in an increased level of mRNA transcript of *sodA* gene in *S*. Enteritidis. To further test the level of production of SodA protein in strains carrying different constructs, western blotting was performed on the total cell lysates of these strains (**Figure 5.2d**). The result showed that production of SodA was reduced in the 654Δ*rnc* strain when compared to the parental strain 654; hence this finding was inconsistent with the observation that this strain produced a large amount of the *sodA* transcript. On the other hand, the quantity of SodA detectable in strain 654Δ*rnc*/p-*rnc* was similar to that of 654. Taken together, the results of qPCR and western blot assays therefore showed that, although deletion of *rnc* gene caused an increase in the amount of mRNA transcript of the *sodA* gene in *Salmonella*, production of the SodA protein was reduced. The level of reactive oxygen species (ROS) in strains carrying these constructs was also determined, with results showing that the ROS level in 654Δ*rnc* was significantly higher than that in 654 and 654Δ*rnc*/p-*rnc*, and comparable to the *sodA* gene knockout mutant 654Δ*sodA* (**Figure 5.2f**). Based on these findings, we conclude that the *rnc* gene played an important role in mediating the translation process of the SodA protein in *S*. Enteritidis and hence ROS metabolism. It should be noted that ROS is a major weapon by which phagocytes utilize to destroy the internalized pathogens. A reduction in the ability to neutralize ROS in *Salmonella* may result in a lower virulence level.

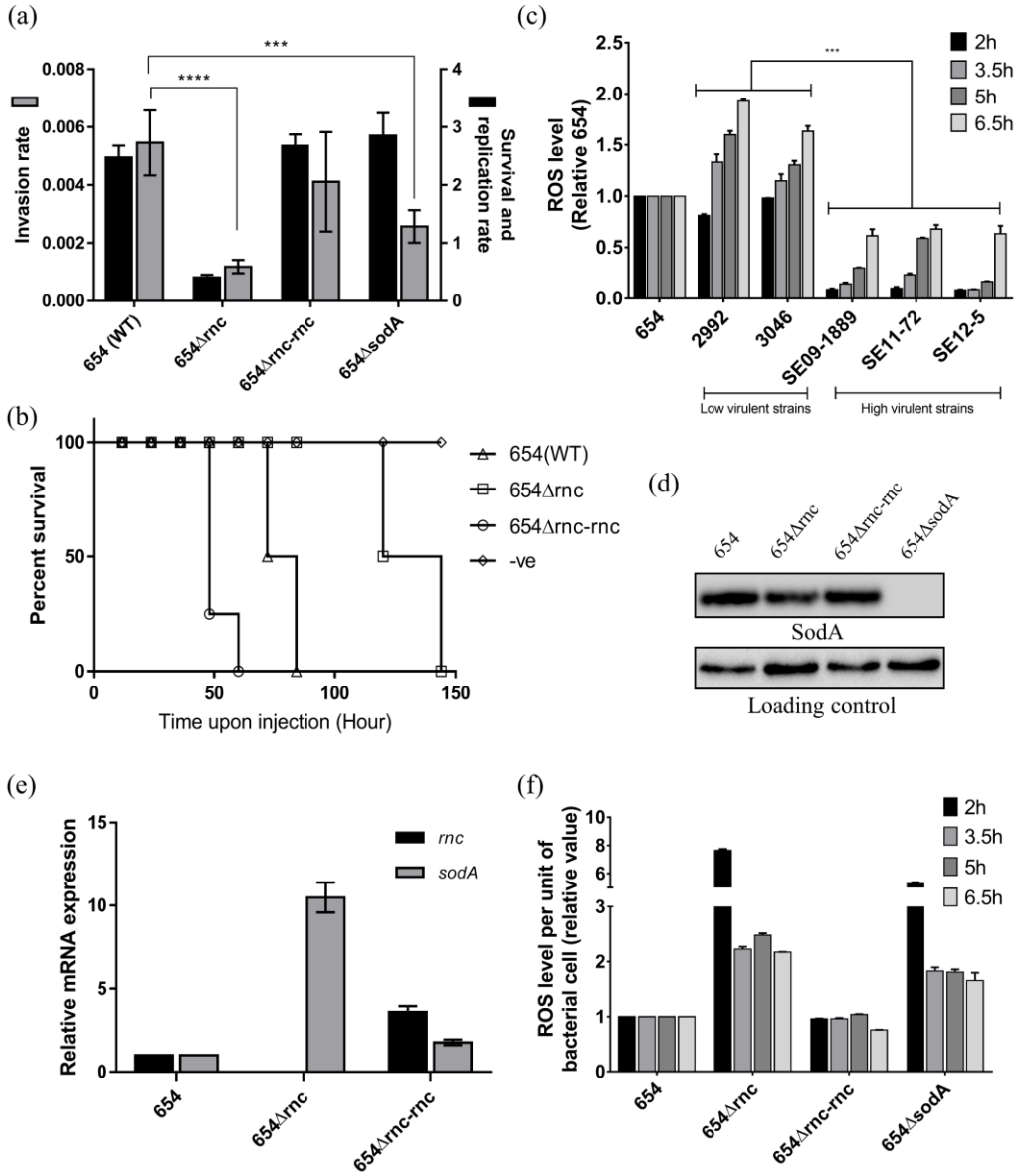

Figure 5.2. Virulence assays of the high virulence food isolate 654 and the corresponding *rnc* gene knockout mutant 654Δ*rnc*. (a)Macrophage internalization and replication assay. (b)Murine sepsis infection assay. The vector pET-*rnc* which carried the *rnc* gene was transformed into strain 654Δ*rnc* to produce 654Δ*rnc*::pET-*rnc,* which was then used in a complementation test.

The superoxide dismutase SodA was responsible for the clearance of reactive oxygen species (ROS) and was regulated by the expression of *rnc*. (c)ROS level in *Salmonella* isolates at different time intervals upon treatment with H_2O_2. (d)Western blot of the SodA protein in strains carrying different constructs. GAPDH was used as endogenous control. (e)The mRNA transcript level of the *sodA* gene in strains carrying different constructs. (e) The level of ROS in strains carrying different constructs.

To further confirm that the reduced virulence of strain 654Δ*rnc* was due to reduced production of SodA, the p-*sodA* vector was transformed into strain 654Δ*rnc* to produce 654Δ*rnc*/p-*sodA*. The *sodA* gene in the vector was over-expressed to produce SodA protein. The RAW264.7 cell invasion and survival and replication assay were carried out to determine the virulence level of strains carrying different constructs (**Figure 5.3a &b**). Deletion of the *rnc* gene from *S*. Enteritidis 654 was found to dramatically lower the invasiveness, as well as the survival fitness and replication rate to level about 1/5 of that of the wild-type strain. The deletion of the *sodA* gene did not affect invasiveness of *Sallmonella* into RAW264.7 cels, while caused a half reduction of survival and replication rate. These results confirm that SodA plays an important role in the survival of *Salmonella* in the macrophage. The transformation of a *rnc*-bearing plasmid to the knockout mutant 654Δ*rnc* restored its intracellular survival and replication rate to the level harbored as its wild-type strain. The intracellular survival and replication rate of strain 654Δ*rnc/*p-*sodA* was about 3-fold that of 654Δ*rnc*, suggesting that introduction of the *sodA* gene can partially restore the function of *rnc*. Similar results were observed in mouse infection assay (**Figure 5.3c**), in which the virulence level of strain 654Δ*rnc*/p-*sodA* was comparable to the parental strain 654; these findings further confirm that reduction in the virulence level of strain 654Δ*rnc* is due to the lowered production of SodA protein.

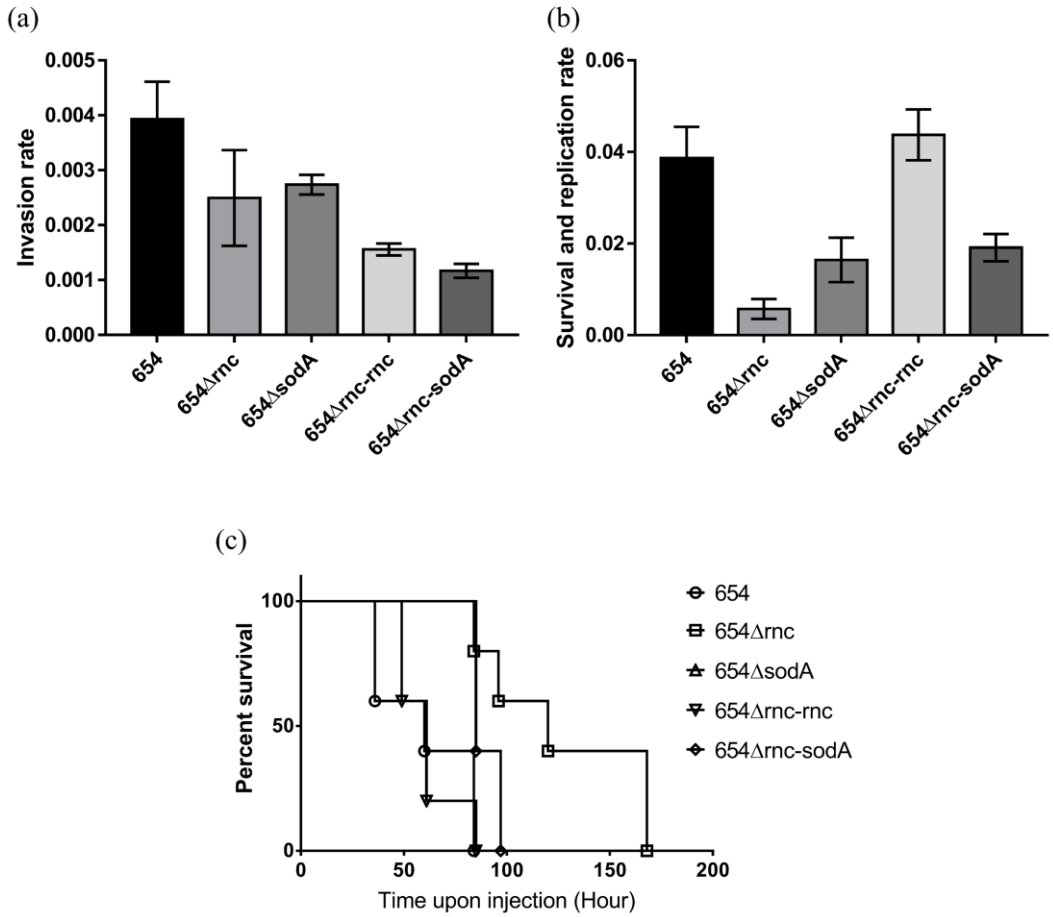

Figure 5.3. Virulence assays of *Salmonella* strains carrying different constructs. (a) RAW264.7 invasion rate of strains carrying different constructs. (b) RAW264.7 intracellular survival and replication rate. (c) Mortality rate of mice at different time points after infection of *Salmonella* strains carrying constructs through tail vein injection.

Double-stranded RNA in Salmonella could induce immune response in the host

Detection of double-stranded RNA is an important criterion of innate immune system in many organisms, especially for defense against viral infection which frequently harbored double-stranded RNA as their genetic material[61]. A known function of the gene product of *rnc* in bacteria is to digest double-stranded RNA (dsRNA). We hypothesized the high expression of *rnc* gene in high virulence *Salmonella* could promote elimination of dsRNA and minimize stimulation of the host immune response during infection. To investigate whether the host immune response could be triggered by dsRNA of *Salmonella*, total RNA extracted from *S.* Enteritids 654 strains carrying different constructs were used to transfect different mammalian cell lines, followed by monitoring the changes in immune signals. Expression levels of TNF-α (**Figure 5.4a)**, IL-1β (**Figure 5.4b**), MDA-5 (**Figure 5.4c**) and IFN-β (**Figure 5.4d**) could be detected in RAW264.7 macrophage cells; on the other hand, expression of IFN-β (**Figure 5.4e**), MDA-5 (**Figure 5.4f**), and RIG-I (**Figure 5.4g**) were detectable in CaCO-2 colon epithelial cells. The results therefore showed that all the immune signals tested were inducible in the RAW264.7 and CaCO-2 cells when they were transfected with total RNA extracted from strains bearing different constructs, suggesting that the RNA from *Salmonella* would stimulate the host immune response. The RAW264.7 cells transfected with total RNA extracted from *S.* Enteritidis strain 654 and 654Δ*rnc*/p-*rnc* exhibited similar expression levels of TNF-α, IL-1β, MDA-5 and IFN-β. However, cells transfected with total RNA from 654Δ*rnc* exhibited significantly higher expression of immune signals; in particular, the level of expression of IL-1β and IFN-β was more than 50% higher than the other transfectants. Similar results were observed among the transfected CaCO-2 cells. For example, expression of IL-1β and RIG-I in CaCO-2 cells transfected with total RNA extracted from strain 654Δ*rnc* was 4-fold that of the cells transfected with total RNA of strain 654 and 654Δ*rnc*/p-*rnc*. The expression level of

MDA-5 in CaCO-2 cells even exhibited 10-fold increase. These findings showed that the excess dsRNA in strain 654Δ*rnc* could strongly induce the immune responses in the RAW264.7 and CaCO-2 cells.

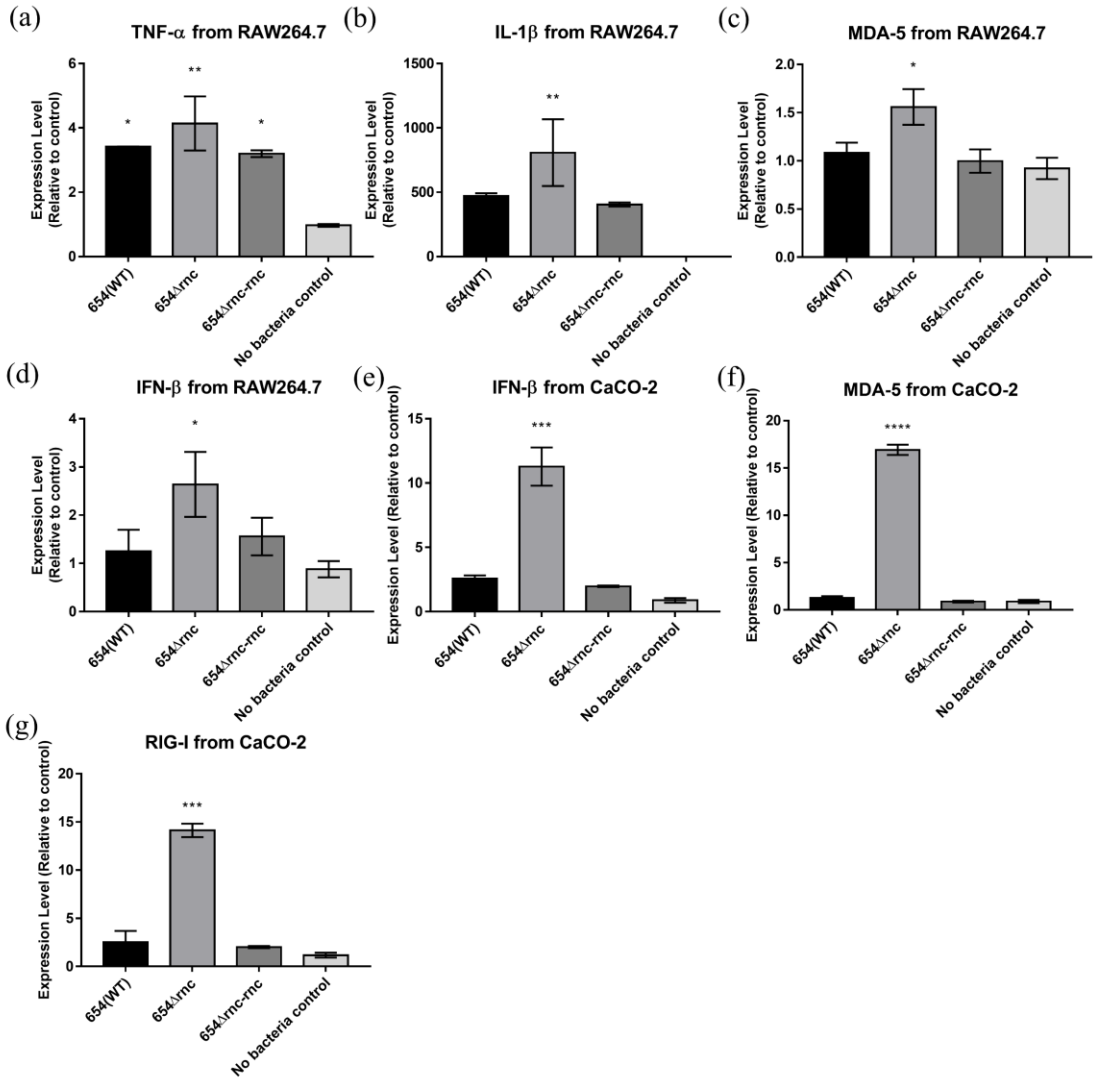

Figure 5.4. Expression of immune signals in macrophage RAW264.7 and colon epithelial cells CaCO-2 upon being transfected by total RNA extracted from *Salmonella* strains carrying different constructs. RNA was transfected into the cell using Lipo2000 transfection medium. (a) Expression of (a)TNF-α, (b)IL-1β, (c)MDA-5, (d)IFN-β in RAW264.7 cell line; Expression of (e)IFN-β, (f)MDA-5, (g)RIG-1 in CaCO-2 cell line. The cells of no-bacteria control were treated with Lipo2000 transfection medium without RNA.

To further confirm that the host immune responses could be triggered by dsRNA of *Salmonella*, total RNA extracted from two clinical isolates, namely SEBL and SEST, and their corresponding *rnc* knockout mutants SEBLΔ*rnc* and SESTΔ*rnc*, were also used to transfect the RAW264.7 macrophage cells and CaCO-2 colon epithelial cells. Consistent with results of experiment involving the 654Δ*rnc* and 654Δ*rnc*/p-*rnc* strains as described above, the mRNA expression level of IFN-β in RAW264.7 transfected with the total RNAs of SEBLΔ*rnc* and SESTΔ*rnc* was 100-fold and 200-fold higher than that transfected with RNAs from SEBL and SEST, respectively (**Figure 5.5a**). Similarly, two-fold difference in mRNA expression level of MDA-5 and RIG-I was observed in cells transfected with RNA extracted from strain SEBLΔ*rnc* and SEBL; for cells transfected with RNA extracted from strain SESTΔ*rnc* and SEST, three-fold difference was observed (**Figure 5.5b, c**). The expression level of IFN-β in CaCO-2 cells was also determined (**Figure 5.5d**), with results showing that transfection with total RNA extracted from strain SEBLΔ*rnc* or SESTΔ*rnc* led to 30-fold over-expression of IFN-β when compared to cells transfected with total RNA extracted from strain SEBL or SEST. All in all, these findings confirm that the total RNA from SEBL and SEST, and the corresponding *rnc* knockout mutants could stimulate the immune response of RAW264.7 and CaCO-2 cells, but expression of the *rnc* gene results in a certain degree of suppression on such stimulatory effect by causing reduction in the amount of dsRNA available for triggering the host immune response, thereby facilitating the infection process.

To further confirm that the uplifted immune responses of RAW264.7 and CaCO-2 cells was triggered by the dsRNA instead of single-stranded RNA (ssRNA) from *Salmonella*, the total RNAs from strain SEBL, SEBLΔ*rnc*, SEST and SESTΔ*rnc* were treated with RNase III and Exonuclease T respectively, followed by transfection into RAW264.7

cells and measurement of expression of IFN-β (**Figure 5.5e**). The result showed that the expression levels of IFN-β in cells transfected with RNase III-treated RNA samples of all test strains were similar. However, total RNA recoverable from SEBLΔ*rnc* and SESTΔ*rnc* treated with exonuclease T could still induce much higher level expression of IFN-β in RAW264.7 cells than strains SEBL and SEST in a manner resembling the no-treatment group. This finding implies that expression of IFN-β in RAW264.7 cells was mainly induced by dsRNA but not ssRNA of *Salmonella*.

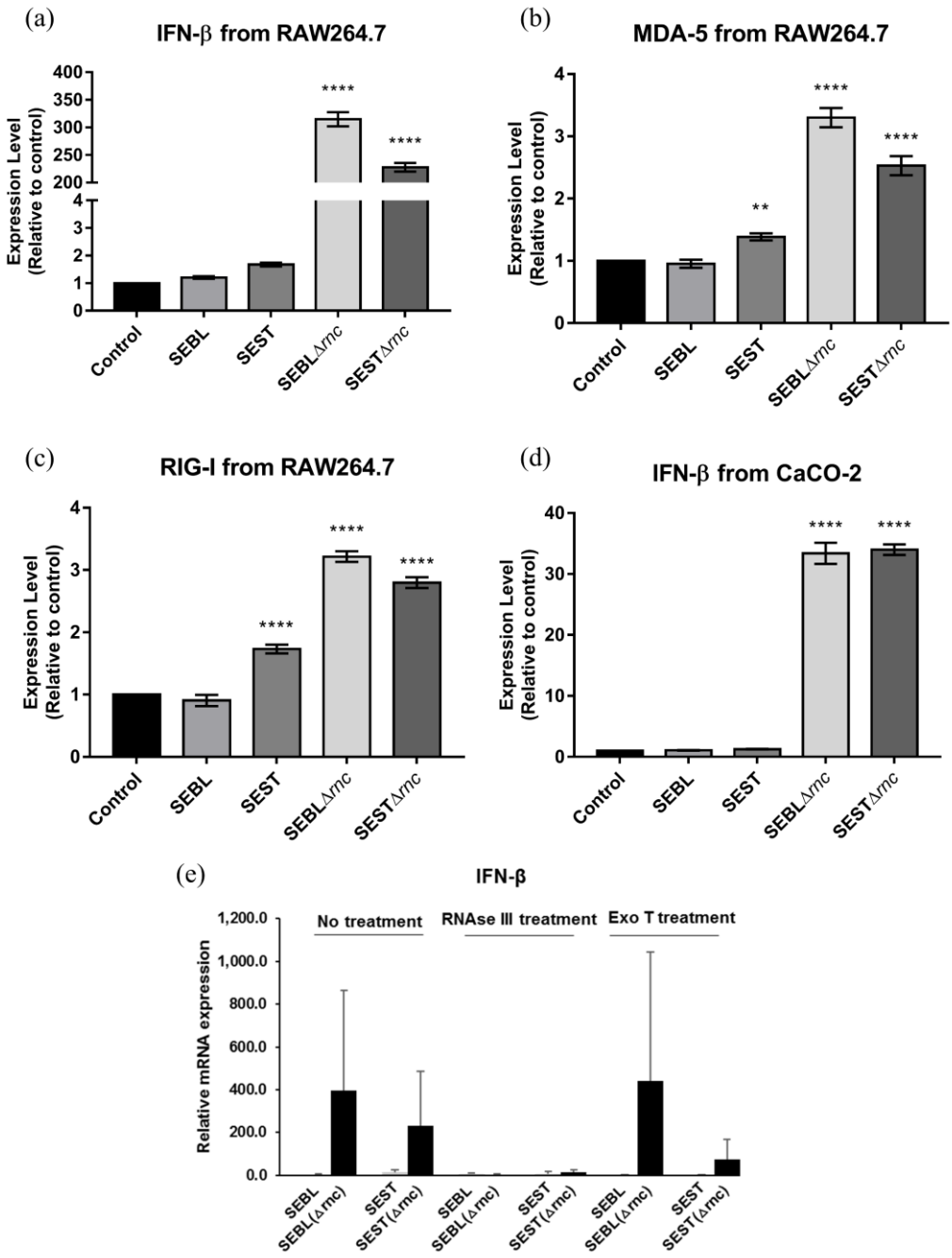

Figure 5.5. Expression of immune signals in macrophage RAW264.7 and colon epithelial cells CaCO-2 upon being transfected by total RNA extracted from clinical *Salmonella* isolates. (a to d) RNAs extracted from strain SEBL and SEST, and their corresponding knockout mutants SEBLΔ*rnc* and SESTΔ*rnc* were transfected into two cell lines. The expression levels of immune signal were detected. (e)Total RNAs of the four *Salmonella* strains were extracted and treated with RNase III (dsRNA endonuclease) and exonuclease T (single-stranded RNA nuclease) respectively, followed by transfection into CaCO-2 cell line to elicit different expression level of IFN-β. The cells treated with Lipo2000 only were used as control.

Discussion

Antisense RNA (asRNA) is known to commonly exist in bacterial genome and can be transcribed from the negative strands of protein-encoding genes. This type of RNA exhibits perfect complementation to RNA transcripts of the sense strand of the gene and form double-stranded RNAs (dsRNAs) by base-pairing with the mRNAs to regulate expression of specific genes[104]. RNase III, a double-stranded RNA (dsRNA)-specific riboendonuclease encoded by the *rnc* gene, is implicated in cleavage of dsRNAs[93]. The molecular mechanisms and biological functions of asRNAs in bacteria are still poorly defined. The widespread presence of asRNAs in bacteria could be transcription noises which are rapidly degraded by RNases; alternatively, these molecules may play a role as gene regulators that fine-tune the expression level of specific genes. Recently, cellular activities that determine the degree of accumulation of dsRNA have been reported to be involved in regulation of both bacterial infectivity and host immune responses[13, 69]. Currently available data also suggest that the functional roles of RNase III in *Salmonella* involve regulation of the level of specific asRNA to control bacterial gene expression; such function, which may be mediated through non-specific regulation of the dsRNA level, may indirectly cause changes in the virulence level of *Salmonella*. We therefore investigated the mechanisms of RNase III in regulating *Salmonella* virulence.

In this study, we first observed that the *rnc* gene which encodes RNase III was over-expressed in the high virulence strains of *Salmonella*. We generated the *rnc* knockout mutant to investigate its role in expression of the virulence phenotype and found that the Δ*rnc* mutant indeed exhibited decreased intracellular survival and replication rate in macrophage cells. Reduction in virulence level in *Salmonella* when *rnc* was deleted was confirmed by testing in murine sepsis model. It is known that *Salmonella* is

exposed to an abundance of ROSs produced by the host during infection, including superoxide (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{3-})[38]. These ROSs were important tools by which the immune system of the host utilize to eradicate pathogens via exerting strong oxidative effects. It was reported in previous studies that the ability of *Salmonella* to neutralize ROS was important for its survival and propagation in the host, and that such function was mediated through production of the enzyme manganese superoxide dismutase (Mn-SOD)[55, 107, 118]. Based on our experimental results and information provided by other studies, we hypothesized that production of Mn-SOD in the Δ*rnc* mutant may be altered. By investigating the expression level of the *sodA* gene which encodes the SodA protein, a kind of Mn-SOD, we found that the transcription of *sodA* gene in the 654Δ*rnc* strain was actually significantly higher than its parental strain 654. However, western blotting experiment showed that the production of SodA protein was significantly reduced in 654Δ*rnc*. These contradictory findings appear to suggest that deletion of *rnc* in *Salmonella* results in reduced RNase III production, which may cause accumulation of dsRNA including that of the transcription product of *sodA*, hence high transcript level of *sodA* was detectable. On the other hand, the asRNA of *sodA* could not be removed due to the lack of RNase III. Hence, the translation of *sodA* transcript would be inhibited, resulting in decreased production of SodA protein. In this study, we performed an ROS assay and confirmed that the ROS content in *Salmonella* significantly increased when the *rnc* gene was deleted (**Figure 5.6**). This finding supports the idea that deletion of the *rnc* gene exhibits the effect of suppressing production of SodA protein production by inhibiting translation of the *sodA* transcript, hence, indirectly affect survival of *Salmonella* in the host body.

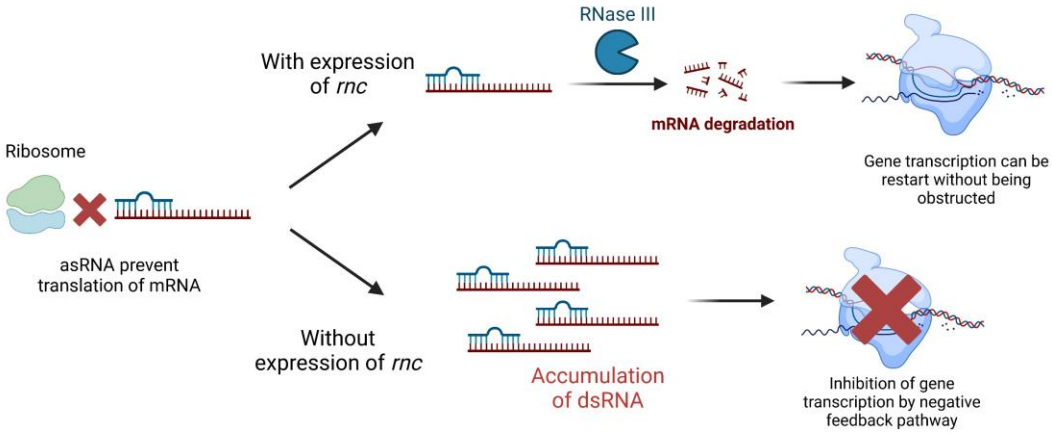

Figure 5.6. Demonstration of gene expression inhibition due to the accumulation of dsRNA.

Apart from demonstrating the role of RNase III in up-regulating expression of the SodA protein, we also comprehensively elucidated the role of RNAse III in regulating the host immune response. First, we found that the RNAs extracted from the *rnc* knockout mutant indeed induced significantly higher levels of expression of immune responses in the host, including the immune molecules IFN- β, RIG-I and MDA-5, when compared to the wild-type strain. Second, we showed that dsRNAs were only detectable in *rnc* knockout mutant, suggesting that bacterial RNAs can be recognized by immune cells and that such RNA molecules can trigger immune response in the host. The retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) were known to be responsible for combating viral infection[59]. Double stranded RNA is commonly found in various types of viruses and is the main component of their genetic materials. The innate immunity elicited against dsRNAs plays an essential role in protecting human against fatal viral infections. Our experimental results showed that the dsRNAs that exist in *Salmonella* could also trigger expression of both RIG-I and MDA-5 pathways. A previous study showed that the alpha/beta interferon-based immune defenses could be triggered by RIG-5 and MDA-5[80]. Our qPCR data showed that expression of IFN-β in RAW264.7 and CaCO-2 cells

significantly increased when the cells were transfected with RNA extracted from the *rnc* knockout mutant. Importantly, testing the effect of treatment by RNase III and Exonuclease T confirmed that such response was only triggered by dsRNAs from *Salmonella* but not ssRNAs. We therefore conclude that the dsRNA from *Salmonella* may act in a manner similar to that of viral dsRNAs by triggering expression of RIG-5 and MDA-5, which in turn activate different immune pathways in the host. Over-expression of RNase III in the high virulence *Salmonella* strains could therefore remove excess dsRNAs and help evade the host immunity.

Conclusion

To conclude, findings in this work suggest that accumulation of dsRNA played an important role in regulating the expression of Mn-SOD, which is important for survival of *Salmonella* in the host during the infection process. On the other hand, we also found that dsRNA was responsible for induction of the host immune response and that RNase III plays a key role in suppressing this induction process. Over-expression of RNase III in *Salmonella* results in a lower dsRNA level, leading to a milder immune response inducible by *Salmonella*. These novel findings pave the way to devise effective approaches to attenuate bacterial virulence by suppressing expression of specific housekeeping and stress response genes, as well as their ability to degrade dsRNA, a key factor that triggers the host immune response.

CHAPTER VI-Conclusion and Recommendation for Future Research

The works described in this thesis revealed the constitutively expressed nature of the plasmid-borne TMQR element *aac(6')-Ib-cr* and *oqxAB*. Further studies are warranted to elucidate the actual events underlying the interaction between the products of *aac(6')-Ib-cr*, *oqxAB*, *oqxR* and *ramA* in *Salmonella* spp., as well as mechanisms regulating the expression of TMQR genes in other members of *Enterobacteriaceae*. Apart from the fluoroquinolone resistance in *Salmonella*, the rapid development of cephalosporins and carbapenem resistance among this species is becoming a global health threat. We should pay more attention to this issue and devise a solution before last resort of antibiotics fail in clinical treatment of bacterial infection. On the other hand, the reduction of virulence level of *Salmonella* by the *oqxABR* locus was demonstrated in this thesis. The lowered virulence may be beneficial to *Salmonella* by allowing this pathogen to better adapt to the animal and human host, as well as facilitating inter-host transmission. However, the molecular interaction between the *oqxABR* and the immune system of host is still not elucidated. Further study shall be conducted to help control the transmission of this opportunistic pathogen.

A unique approach was adopted in this thesis to study *Salmonlla* pathogenesis, through which we discovered that a wide range of genes that encode regulatory and metabolic functions played an important role in mediating expression of virulence phenotypes in *Salmonella*. The two important global regulatory genes, namely *yhjB* and *yaiV*, was found to play a role in regulating multiple secondary regulator genes that in turn control expression of virulence in *S.* Enteritidis. This finding provides novel insight into how *Salmonella* virulence is regulated at the transcriptional level. On the other hand, dsRNA was found to play an important role in regulating the expression of Mn-SOD and induction of the host immune response. Over-expression of RNase III in *Salmonella*

plays a key role in suppressing this induction process, resulting in milder immune response inducible by *Salmonella*. These novel findings pave the way to devise effective approaches to attenuate bacterial virulence by suppressing expression of specific housekeeping and stress response genes, as well as their ability to degrade dsRNA, a key factor that triggers the host immune response.

However, the exact function and genetic elements behind these regulatory networks are still poorly defined, suggesting a need to conduct further studies to identify the key virulence regulatory genes in the high virulence *Salmonella* strains in different environmental niches, and investigate why such regulators are less actively expressed in the low virulence strains despite the fact that they are genetically identical.

Studies described in this thesis provide much novel insight into development of effective strategies to combat invasive *Salmonella* infection, especially the typhoidal *Salmonella* strains which pose enormous threat to human lives in developing countries.

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