KEY MESSAGES

1. The virulence of Salmonella is not defined by the genetic traits of specific strains but by the expression of specific house-keeping and stress-response genes, including HilA, HilD, HilC, and RNase III.
2. Salmonella double-stranded RNA (dsRNA) can induce host immune responses.
3. The RNase III level defines the ability of a Salmonella strain to trigger host immune response.
4. Over-expression of RNase III occurs in the highly virulent strains Salmonella, resulting in a lower dsRNA level and hence milder immune response and higher virulence.

Introduction

To infect, bacteria must replicate and produce a complex array of gene products for invasion and survival in the host environment. Microorganisms frequently undergo adaptive physiological and genomic changes; bacterial virulence is subjected to constant regulatory pressure characteristic of the nature of environmental stress that the organisms encounter. Salmonella express a repertoire of known virulence factors and undergo physiological phase transition upon traversing between the natural environment and host body, encountering and overcoming stresses of different nature. Our preliminary study indicated that strains of the same serotype often exhibit a spectrum of phenotypic characteristics such as survival fitness in macrophages. We hypothesise that Salmonella constantly generates mutational changes for environmental adaptation in such a way that only a subset of the environmental organisms is physiologically fit to infect humans. This study aimed at analysing the genetic backgrounds and gene expression profiles of organisms that exhibit high and low virulence potential under clinically relevant conditions. Findings can provide invaluable insight into the key regulatory and functional mechanisms that define the virulence level of Salmonella.

Methods

Salmonella enteritidis strains (30 clinical samples, 5 food samples, and 26 Centers for Disease Control and Prevention samples) were used in this study.

Salmonella typhimurium LT2 was used as a reference strain.

DNA was extracted from overnight culture of the test organisms using PureLink Genomic DNA Mini kit (Invitrogen) and used for PCR-virulotyping and whole genome sequencing. Eleven known virulence determinants were tested: invA, avaR, ssaQ, mgaC, sipD, sipB, gipA, sodC, sopE, spvC, and bcfC.

The virulence of Salmonella strains was characterised by infecting RAW 364.7 cells. Internalisation rate and replication rate were calculated as follows: 25 µL of bacterial suspension were added to the wells at a multiplicity of infection of 10:1, followed by incubation at 37°C, 5% CO2 for 25 minutes; the plates were then washed twice with pre-warmed PBS and incubated with media containing 100 µg/mL gentamicin for 1 h. The supernatant was then removed at 2 and 16 h after infection; the cells were then washed twice with pre-warmed PBS and lysed with 0.1% Triton X-100. Serial dilutions of the lysates (10⁻¹, 10⁻², 10⁻³, 10⁻⁴) were then plated onto LB agar to enumerate the intracellular bacteria.

Invasion rate or internalisation rate (bacteria number to infect or intracellular CFU after 2 h per well after infection) was used to describe the invasion ability of isolates. Replication rate (intracellular CFU after 2 h or intracellular CFU per well after 16 h) of the test isolates was also recorded.

To detect the potential virulence related mutagenesis or emergence of novel virulence genes, whole genome sequencing of selected strains was conducted on the Illumina platform. To depict the
expression profiles of different virulence-related genes, transcriptome analysis of high and low virulence was performed. The strains were harvested in LB broth with 0.6 of OD600, and the total RNA was extracted using RNeasy Mini Kit and proceed to perform RNA-seq in BGI Company in Hong Kong.

To investigate the functions of genes, mutant strains were constructed using the RED-mediated recombination system reported.5

Overnight *Salmonella* cultures were diluted 100x in LB broth and cultured for 3 and 12 h at 37°C. The bacteria were collected, and total RNA was isolated by the RNA isolation kit (Qiagen). Bacterial total RNA was quantified, and similar amount of RNA was separated on agarose gel, transferred to PVDM membrane, and detected using J2 antibody.

RAW264.7 cell was cultured in DMEM. Overnight cell culture was transfected with total RNA with or without Lipo2000 transfection reagent based on the manufacturer instructions. The expression of different immune factors was measured by qRT-PCR.

Results
We first investigated if food and clinical isolates of *S enteritidis* harboured different virulence gene profiles. Based on the prevalence of virulence-related genes in 61 *S enteritidis* isolates, we did not observe significant difference in the distribution of virulence genes among these isolates, suggesting that *S enteritidis* strains that originated from different sources exhibited a similar level of virulence.

We then examined if the strains exhibited differential virulence levels despite their similar genetic profiles. Strains of the highest and lowest virulence levels were determined by their invasion capability and survival rate in macrophages. We found that genetically identical strains may exhibit highly different virulence levels (Fig 1). The lowest and highest relative invasion rates of the test isolates were 0.0009 and 0.3863, respectively, a difference of >400 fold. For replication rate, the lowest and highest were 0.0348 and 15.3199, respectively.

To delineate the cellular basis of differential virulence levels observable in genetically identical organisms, strains exhibiting the lowest and highest virulence were subjected to RNA-Seq. The high virulence group of *S enteritidis* consistently displayed higher levels of expression of virulence-related and host adaptation-related genes than the low virulence group. In particular, expression of specific two-component system (PhoQ), carbon storage regulator (CsrA), invasion protein regulator (HilA), DNA-binding protein Fis, *araC* family transcriptional regulator (HilD and HilC), RNase III and other elements with less definitive functions such as SicA were found to be dramatically increased in high virulence group but not in the low virulence group.
The *rnc* gene, which encodes RNase III, was also found to play a particularly important role in *Salmonella* virulence (Fig 2). In a mobility assay, two *rnc* knockout strains, S1::Δ*rnc* and S3::Δ*rnc*, were found to exhibit significantly reduced mobility, suggesting that *rnc* is essential for *Salmonella* mobility. In addition, deletion of the *rnc* gene also resulted in insignificant impairment of the virulence of *Salmonella* in a wax worm model, thus further confirming the important role of *rnc* in *Salmonella* virulence.

We then tested whether *Salmonella* strains S1 and S3 and the respective *rnc* knockout strains could induce immune response. The expression level of IFN-β increased with the increasing multiplicity of infection. In addition, *rnc* knockout strains were found to exhibit higher levels of expression of IFN-β, compared with the wild type S1 and S3 strains (Fig 3). We also tested whether the role of RNase III in inducing immune response is due to its ability to control formation of dsRNA in *Salmonella*. Double-strand RNA assay showed that dsRNAs were only detectable in S1::Δ*rnc* and S3::Δ*rnc*, but not in the wildtype strains, suggesting that RNase III indeed plays an important role in cleaving endogenous dsRNAs in *Salmonella*.

To check whether *Salmonella* dsRNA was involved in bacterial infection and host immune response, we performed dsRNA transfection assay in RAW264.7 cells. After 16 h transfection, bacterial RNA from *rnc* knockout strains, but not those of the wild type strains, caused over-expression of IFN-β. Induction of IFN-β may be mediated by the over-expression of RIG-I and MDA5 receptors, which were also inducible by *Salmonella* RNA. These findings suggest that bacterial RNAs could trigger the immune response.

**Discussion**

We presented an overview of the *Salmonella* virulence profile at both genetic and gene expression level and the underlying basis of high virulence in specific strains. The genetic and virulence gene profiles of all test strains of *S. enteriditis* were highly similar, suggesting that any difference in virulence level between individual strains is not due to a discrepancy in the virulence gene profile. Nonetheless, virulence assays that tested the ability of *Salmonella* to invade and replicate in macrophages showed that the invasiveness of individual strains could differ significantly, despite their almost identical virulence gene profiles. The high-level virulence in *S. enteritidis* was associated with up-regulation of many virulence-related and host adapted related genes, as well as a number of global regulators such as those encoding PhoQ, CsrA, HilA, and RNase III. These findings suggest that the pathogenicity of *Salmonella*, or perhaps...
other bacterial pathogens, is highly dependent on the transcription status of a wide range of intrinsic regulatory and functional genes that are present in all strains. Such findings imply that acquisition of new virulence genes may only play a rather minor role in fine-tuning the basal virulence level of the organism.

Of particular importance is the rnc gene, which is known to encode RNase III and over-expressed in high virulence Salmonella strains. We elucidated the role of RNase III in regulating the host immune response, and hence, indirectly, survival of the pathogen in the host body. The rnc knockout strain was less virulent and exhibited lower mobility. rnc knockout strains indeed exhibited higher levels of expression of IFN-β. dsRNAs were only detectable in rnc knockout strains. Bacterial RNAs could be recognised by immune cells, triggering the immune response. Therefore, our data confirm that dsRNA is responsible for the induction of the host immune response and that RNase III plays a key role in regulating such induction potential. Over-expression of RNase III in Salmonella is expected to result in a lower dsRNA level, leading to a milder immune response inducible by Salmonella. These findings may help devise novel strategies to attenuate bacterial virulence by suppressing expression of specific house-keeping and stress response genes, as well as their ability to degrade dsRNA, a trigger of host immune response.

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**Disclosure**

The results of this research have been previously published in:


**References**