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石河子大学

博 士 学 位 论 文

sRNA *STnc3020* 对鼠伤寒沙门菌 T3SS 分泌装置形成的调控作用及分子机制研究

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研 究 方 向　动物生产与疾病控制
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Exploration of the Regulatory Role and Molecular Mechanisms Underlying of sRNA *STnc3020* on the Assembly of the T3SS Secretory Apparatus in *Salmonella* typhimurium

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By

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(Animal production and disease control)

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June, 2025

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摘 要

鼠伤寒沙门菌（*Salmonella typhimurium*，*S. typhimurium*）为革兰氏阴性，兼性胞内寄生菌，是一种重要的人兽共患病原菌。该菌主要通过粪口途径传播，感染人类时常引发胃肠炎和败血症，而在动物中则可导致急性胃肠炎及怀孕母畜流产。目前，*S. typhimurium* 在食品中的污染日益严重，已成为食源性感染的主要病原体，严重威胁公众健康。*S. typhimurium* 能够在多种宿主环境中生存并繁殖，且易形成生物膜，这使得它能够引起不同的疾病，从自限性胃肠炎到全身性感染，导致较高发病率和死亡率。非编码小 RNAs（noncoding small RNA，sRNA）是一种长度为 40~500 个核苷酸，是转录后水平调控基因的表达调控元件。生物信息学分析发现 *STnc3020* 为 *S. typhimurium* 潜在 sRNA，RT-qPCR 检测发现其在感染巨噬细胞时高表达，但具体的生物学功能及调控机制暂不清楚。研究方法和主要研究结果如下：

1. 鼠伤寒沙门菌感染巨噬细胞后高表达 sRNA 的筛选、鉴定、基因组定位及分子特征分析

利用在线软件预测 S. *typhimurium* 基因组中潜在 sRNA，对其基因组定位、启动子长度分布、二级结构、靶基因及其启动子区进行分析。同时，提取 S. *typhimurium* 感染小鼠巨噬细胞（RAW264.7）后的总 RNA，利用 RT-qPCR 检测部分 sRNA 在巨噬细胞内外的表达。预测结果显示，在 *S. typhimurium* 中共有 280 个潜在 sRNA，其中 sRNA *STnc3020*、*STnc1410*、*STnc4240* 及 *InvR* 位于沙门菌毒力岛 1（*Salmonella* pathogenicity islands，SPI-1），*IsrC*、*STnc3420* 和 *STnc520* 位于 SPI-11，*STnc1220* 位于 SPI-2，*AmgR* 位于 SPI-3 及 *IsrA* 位于 SPI-6 等。分子特征分析结果显示，280 个 sRNA 长度在 40-500 nt 之间，对 sRNA *STnc3020*、*STnc270*、*STnc1410*、*STnc520*、*GcvB*、*STnc1220*、*STnc3090* 及 *STnc3080* 进行二级结构预测，发现以上 8 个 sRNA 均具有典型的茎环结构，这些结构在 sRNA 的稳定性、靶标结合和功能调控中发挥重要作用。RT-qPCR 检测结果发现，在感染巨噬细胞后 sRNA *STnc3020*、*STnc3080* 和 *STnc3090* 表达量上调，这表明这些 sRNA 在 *S. typhimurium* 感染宿主细胞的过程中，可能发挥着重要的调节作用。基于以上结果，选择了位于 SPI-1 区域且在感染巨噬细胞时高表达的 sRNA *STnc3020* 作为进一步研究对象，以深入探讨其在 *S. typhimurium* 感染过程中的具体功能和调控机制。

2. sRNA *STnc3020* 缺失株、回补株和示踪菌的构建及致病性分析

以 *S. typhimurium* SL1344 为研究对象，用λ-red 同源重组技术构建 *STnc3020* 缺失株（*ΔSTnc3020*）和回补株（C*ΔSTnc3020*）。随后，将含有红色荧光的 pFcCGi 质粒电转

入 SL1344、*ΔSTnc3020* 和 C*ΔSTnc3020* 菌株中，获得了带有红色荧光标记的示踪菌株，并对这些菌株进行了生长能力和表面形态的分析。同时，以构建的各菌株为对象，通过小鼠感染模型，评估各菌株对小鼠的半数致死量（LD_{50}）、在肝脏、脾脏和盲肠中的载菌量、盲肠炎性因子、脏器病理组织学分析及感染分布等。PCR 和测序验证结果显示，成功构建*ΔSTnc3020* 和 C*ΔSTnc3020* 菌株，荧光显微镜观察结果证实示踪菌株构建成功。小鼠感染模型结果显示：*ΔSTnc3020* 感染小鼠的 LD_{50} 值增加，并且其在肝脏和盲肠中的定殖量减少，炎性因子 IL-6 和 IFN-γ的水平下降。组织免疫荧光结果检测发现 SL1344、*ΔSTnc3020*C*ΔSTnc3020* 菌株感染小鼠后主要定殖肝脏、脾脏和盲肠中。以上结果发现，*STnc3020* 缺失影响 *S. typhimurium* 感染小鼠时的毒力作用，表明其在 *S. typhimurium* 致病性中具有一定的调控作用。

3. sRNA *STnc3020* 缺失对鼠伤寒沙门菌感染宿主细胞的影响

将 SL1344、*ΔSTnc3020* 和 C*ΔSTnc3020* 菌株感染小鼠小肠上皮细胞 MODE-K，检测黏附和侵袭能力、肠上皮细胞膜褶皱的形成以及肠紧密连接蛋白的表达水平。同时，感染小鼠巨噬细胞 RAW264.7，检测各菌株在巨噬细胞内增殖、诱导巨噬细胞凋亡及吞噬的能力等。此外，利用蓝色葡聚糖 2000 检测各菌株对小鼠小肠的渗透性。结果表明，与 SL1344 和 C*ΔSTnc3020* 菌株相比，*ΔSTnc3020* 菌株对小鼠肠上皮细胞的黏附的能力及对小鼠小肠的渗透显著降低（$P<0.01$），侵袭能力有所降低，但差异不显著。在感染小鼠肠上皮细胞后，三种菌株均导致细胞膜褶皱和细胞骨架改变，肠紧密连接蛋白表达量发生变化。巨噬细胞感染结果显示：*ΔSTnc3020* 菌株在胞内的增殖率显著低于 SL1344 和 C*ΔSTnc3020*（$P<0.01$）。流式细胞术检测发现 SL1344 和 C*ΔSTnc3020* 菌株诱导的凋亡细胞数量显著多于*ΔSTnc3020* 菌株（$P<0.01$）。Western blot 分析显示，巨噬细胞凋亡蛋白 Bax 在 SL1344 和 C*ΔSTnc3020* 中显著上调，而抗凋亡蛋白 Bcl-2 在*ΔSTnc3020* 中显著升高。这些结果表明，*STnc3020* 在 *S. typhimurium* 感染宿主肠上皮细胞及在巨噬细胞内的增殖和诱导凋亡过程中发挥着具有重要的调控作用，进一步阐明了 *STnc3020* 在 *S. typhimurium* 与宿主细胞相互作用中的调控功能。

4. 鼠伤寒沙门菌 SL1344 野毒株与*ΔSTnc3020* 间的差异表达基因筛选分析

对 *S. typhimurium* SL1344 野毒株与 *STnc3020* 缺失株进行转录组测序（Transcriptome Sequencing，RNA-Seq）分析，利用基因本体（GO）和京都基因与基因组百科全书（KEGG），对差异表达基因参与的生物学过程和信号通路进行分析。测序结果显示，在 SL1344 野毒株与*ΔSTnc3020* 之间有 2572 个差异表达基因，其中 2571 个基因表达下调，1 个基因表达上调。下调基因包括：*prgJ*、*invG* 及 *invI* 等 T3SS 相关基因；毒力因子 *srfA*、*sifA* 和 *sipA*；鞭毛相关基因 *flgA* 和 *flgB*，上调基因为 *fimZ*。GO 功能注释结果显示，*STnc3020* 基因缺失后下调基因主要富集在生物过程和分子功能中。KEGG 通路分析发现，*STnc3020* 基因缺失后下调基因显著富集的代谢通路包括不同环境中的微生物代谢、ABC

转运系统、双组分系统、丙酮酸代谢等。选取的部分差异表达基因 RT-qPCR 验证结果与转录组分析结果一致，为后续研究提供了可靠依据。

5. sRNA *STnc3020* 调控潜在靶基因的筛选、验证及其对 T3SS 装置关键蛋白的调控机制分析

利用细菌双质粒报告系统验证 *STnc3020* 与靶基因 mRNA 的相互作用，并通过结合位点突变进一步验证。此外，用 Western blot 分析 *STnc3020* 对靶基因蛋白表达的影响。同时，提取并纯化 T3SS 分子装置（在体外模拟巨噬细胞内环境），利用透射电镜观察其结构，并检测 T3SS 效应蛋白和总体分泌蛋白的表达。预测结果显示，*STnc3020* 第 3~23 位碱基可以与 *prgJ* mRNA 5'-UTR 的-58~-78 区域互补配对。双质粒报告系统验证结果表明，*STnc3020* 和 *prgJ* mRNA 5'-UTR 相互作用。Western blot 结果表明，*STnc3020* 缺失株中靶蛋白 PrgJ 的表达量明显低于 SL1344 和 C*ΔSTnc3020* 菌株（P<0.01）。分泌蛋白 SDS-PAGE 电泳显示，*ΔSTnc3020* 菌株的总体分泌蛋白水平低于 SL1344；Western blot 结果表明，*ΔSTnc3020* 菌株中 SopE 和 SipC 蛋白的表达量下调，而 SopD 蛋白表达量无明显变化。透射电镜观察结果显示，*ΔSTnc3020* 菌株中 T3SS 分子装置结构不完整，仅能观察到针状结构，而 SL1344 菌株的 T3SS 装置完整。这些结果表明 *STnc3020* 通过转录后水平调控 PrgJ 蛋白的表达，影响 T3SS 的完整性和功能，从而在 *S. typhimurium* 致病机制中发挥重要的调控作用。

总之，本研究系统分析了 *STnc3020* 缺失对 *S. typhimurium* 黏附和侵袭上皮细胞的能力，以及在巨噬细胞内的增殖和诱导巨噬细胞凋亡的能力。同时，本研究还评估了 *STnc3020* 缺失对 *S. typhimurium* 毒力的影响。通过转录组测序、细菌双质粒报告系统、qRT-PCR 和 Western blot 等多种方法，成功鉴定了 *STnc3020* 调控的靶基因。揭示了 *STnc3020* 通过转录后水平调控 T3SS 内杆蛋白 PrgJ 的表达，影响 T3SS 分泌系统的结构蛋白和效应蛋白的表达水平，从而维持 T3SS 分子装置的完整性和功能性。这些发现为进一步深入解析 sRNA 介导的毒力调控机制提供了重要线索和理论依据。

关键词：鼠伤寒沙门菌；sRNA *STnc3020*；毒力；T3SS 分泌装置；调控机制

Abstract

Salmonella typhimurium, (*S. typhimurium*) is a Gram-negative, facultative intracellular parasite and a significant zoonotic pathogen. It is primarily transmitted via the fecal-oral route, causing gastroenteritis and septicemia in humans, and acute gastroenteritis and abortion in pregnant animals. Currently, contamination of food by *S. typhimurium* is increasingly severe, making it a major causative agent of foodborne infections and a serious threat to public health. *S. typhimurium* can survive and proliferate in diverse host environments and readily forms biofilm. These capabilities enable it to cause a spectrum of diseases, ranging from self-limiting gastroenteritis to systemic infections, resulting in high morbidity and mortality. Noncoding small RNAs (sRNAs), ranging from 40 to 500 nucleotides in length, are post-transcriptional regulatory elements that control gene expression. Bioinformatics analysis identified *STnc3020* as a potential sRNA in *S. typhimurium*. RT-qPCR analysis revealed its high expression during macrophage infection; however, its specific biological functions and regulatory mechanisms remain unclear. The research methods and key findings are described below:

1. Screening, identification, genomic localization, and molecular characterization of sRNA highly expressed in macrophages infected by *S. typhimurium*

Using online software, potential sRNA within the *S. typhimurium* genome were predicted, followed by analyses of their genomic localization, length distribution, secondary structures, and target genes. Total RNA was extracted from RAW264.7 macrophages infected with *S. typhimurium*, and the expression of selected sRNA both inside and outside the macrophages was analyzed using RT-qPCR. The prediction results indicated that there are a total of 280 potential sRNA in *S. typhimurium*, among which sRNA *STnc3020*, *STnc1410*, *STnc4240*, and *InvR* are located within *Salmonella* pathogenicity island 1 (SPI-1), *IsrC*, *STnc3420*, and *STnc520* are found in SPI-11, *STnc1220* is in SPI-2, *AmgR* is located in SPI-3, and *IsrA* is situated in SPI-6. Molecular characterization analysis revealed that the lengths of the 280 sRNA range from 40-500 nucleotides. Secondary structure predictions were performed for sRNA *STnc3020*, *STnc270*, *STnc1410*, *STnc520*, *GcvB*, *STnc1220*, *STnc3090*, and *STnc3080*, showing that these eight sRNA possess typical stem-loop structures, which play crucial roles in the stability, target binding, and functional regulation of sRNA.

RT-qPCR results demonstrated that the expression levels of sRNA *STnc3020*, *STnc3080*, and *STnc3090* were upregulated following infection of macrophages, suggesting that these

sRNA may have regulatory roles during *S. typhimurium* infection of host cells. Based on these findings, we selected the sRNA *STnc3020*, located in the SPI-1 region and highly expressed during macrophage infection, as the focus of further investigation to explore its specific functions and regulatory mechanisms in the process of *S. typhimurium* infection.

2. Construction of *STnc3020* deletion strains, complementary strains, and tracer strains, and pathogenicity analysis

Using *S. typhimurium* SL1344 as the research subject, we employed λ-red homologous recombination technology to construct *STnc3020* deletion strains (*ΔSTnc3020*) and complementary strains (C*ΔSTnc3020*). Subsequently, a plasmid containing a red fluorescent protein (pFcCGi) was electroporated into SL1344, *ΔSTnc3020*, and C*ΔSTnc3020* strains to obtain tracer strains. The growth capacity and surface morphology of these strains were analyzed. Next, we utilized a BALB/c mouse infection model to evaluate the pathogenicity of the constructed strains, determining the median lethal dose (LD_{50}) for each strain, as well as the bacterial load in the liver, spleen, and cecum, the levels of inflammatory factors in the cecum, and conducting histopathological analyses of the organs. PCR and sequencing validation confirmed the successful construction of the *ΔSTnc3020* and C*ΔSTnc3020* strains, while fluorescence microscopy confirmed the successful construction of the tracer strains. Growth characteristic analysis demonstrated that the deletion of *STnc3020* and the expression of the fluorescent protein did not affect the growth of the strains. Gram staining and transmission electron microscopy revealed no significant differences in the surface morphology of *ΔSTnc3020* and C*ΔSTnc3020* compared to SL1344.

Results from the mouse infection model indicated that the LD_{50} of ΔSTnc3020-infected mice increased, along with a reduced colonization in the liver and cecum, and decreased levels of inflammatory factors IL-6 and IFN-γ. Immunofluorescence detection further confirmed a reduced distribution of the *ΔSTnc3020* strain within the organs. These findings suggest that *STnc3020* influences the virulence of *S. typhimurium* during mouse infection, indicating its regulatory role in bacterial pathogenicity. This discovery provides an important theoretical basis for further understanding the role of *STnc3020* in pathogen survival and host-pathogen interactions.

3. The effect of sRNA *STnc3020* deletion on the Infection of host cells by *S. typhimurium*

To investigate the impact of *STnc3020* on the infection of host cells by *S. typhimurium*, this study utilized SL1344, *ΔSTnc3020*, and C*ΔSTnc3020* strains to infect mouse intestinal epithelial cells (MODE-K). The adhesion and invasion capabilities, intestinal permeability in

mice, expression of intestinal epithelial cell membrane ruffles, and tight junction proteins were assessed. Additionally, RAW264.7 mouse macrophages were infected to evaluate the proliferation of each strain within macrophages, their effect on inducing macrophage apoptosis, and phagocytic capacity. The results demonstrated that, compared to SL1344 and C*ΔSTnc3020* strains, the *ΔSTnc3020* strain showed significantly reduced adhesion to mouse intestinal epithelial cells and decreased permeability in the mouse intestine ($P<0.01$). While invasion ability was also reduced, the difference was not statistically significant. Following infection of mouse intestinal epithelial cells, all three strains induced membrane ruffling and cytoskeletal alterations, accompanied by a decrease in the expression of tight junction proteins.

In macrophage infection assays, the proliferation rate of the ΔSTnc3020 strain was significantly lower than that of SL1344 and C*ΔSTnc3020* strains ($P<0.01$). Furthermore, the number of apoptotic cells induced by SL1344 and CΔSTnc3020 strains was significantly higher than that of the *ΔSTnc3020* strain ($P<0.01$). Western blot analysis revealed that the pro-apoptotic protein Bax was significantly upregulated in the SL1344 and C*ΔSTnc3020* strains, while the anti-apoptotic protein Bcl-2 was significantly elevated in the *ΔSTnc3020* strain. These results indicate that *STnc3020* plays a crucial regulatory role in the processes of *S. typhimurium* infection of host intestinal epithelial cells, as well as in the proliferation and apoptosis induction in macrophages. This further elucidates the functional role of *STnc3020* in the interactions between pathogens and host cells.

4. Transcriptomic analysis of *S. typhimurium* SL1344 wild-type and *STnc3020* deletion strains and screening of differentially expressed genes

This study conducted transcriptome sequencing (RNA-Seq) to analyze the differences between the wild-type *S. typhimurium* SL1344 strain and the *STnc3020* deletion strain. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to investigate the biological processes and signaling pathways associated with the differentially expressed genes. Additionally, quantitative reverse transcription PCR (RT-qPCR) was employed to validate a subset of differentially expressed genes, ensuring the reliability and accuracy of the results. The sequencing results revealed a total of 2,572 differentially expressed genes between the SL1344 wild-type and *STnc3020* deletion strains, of which 2,571 genes were downregulated and 1 gene was upregulated. The downregulated genes included T3SS-related genes such as *prgJ*, *invG*, and *invI*, virulence factors like *srfA*, *sifA*, and *sipA*, as well as flagellar genes *flgA* and *flgB*, and SPI-6-related genes *sciA*, *sciB, sciC*, *sciD*, *sciE*, and *sciG*. The upregulated gene was *fimZ*.

GO functional annotation indicated that the downregulated genes following the deletion of the *STnc3020* gene were mainly enriched in biological processes and molecular functions. KEGG pathway analysis highlighted that the downregulated genes significantly enriched in metabolic pathways included microbial metabolism in diverse environments, ABC transporters, two-component systems, and pyruvate metabolism. To validate the reliability of the transcriptomic data, selected differentially expressed genes were subjected to RT-qPCR, and the results were consistent with the transcriptomic analysis, providing a solid foundation for subsequent research.

5. Screening and verification of potential target genes regulated by sRNA *STnc3020* and analysis of Its regulatory mechanism on key proteins of the T3SS apparatus

Through bioinformatics analysis combined with RNA sequencing (RNA-Seq), potential target genes regulated by *STnc3020* were identified. A bacterial dual-plasmid reporter system was employed to validate the direct interaction between *STnc3020* and the mRNA of the target gene, with further confirmation achieved through binding site mutations. The impact of *STnc3020* on the protein expression of target genes was analyzed using Western blotting. Additionally, the expression levels of T3SS effector proteins SopE, SipC, and SopD were assessed by Western blot, while the T3SS molecular apparatus was extracted and purified for structural observation using transmission electron microscopy.

Prediction results indicated that the bases 3-23 of *STnc3020* could complementarily pair with the bases -58 to -78 of the 5′-UTR of the T3SS inner rod protein *prgJ* mRNA. The validation using the dual-plasmid reporter system confirmed the direct binding of *STnc3020* to the *prgJ* mRNA 5′-UTR, and this finding was corroborated by binding site mutations. Western blot results demonstrated that the expression level of the target protein prgJ in the *ΔSTnc3020* strain was significantly lower than that in the SL1344 and C*ΔSTnc3020* strains ($P<0.01$). SDS-PAGE analysis of secreted proteins revealed that the overall level of secreted proteins in the *ΔSTnc3020* strain was lower than that in the wild-type SL1344 strain. Western blot results further indicated that the expression levels of SopE and SipC proteins were downregulated in the *ΔSTnc3020* strain, while there was no significant change in the expression level of SopD. Transmission electron microscopy observations showed that the T3SS molecular apparatus in the *ΔSTnc3020* strain was structurally incomplete, with only needle-like structures observed, while the T3SS apparatus in the SL1344 strain appeared intact. These results suggest that *STnc3020* regulates the expression of *prgJ*, thereby affecting the levels of T3SS effector proteins and, consequently, the integrity and functionality of the

T3SS. This underscores the important regulatory role of *STnc3020* in the pathogenic mechanism of *S. typhimurium*.

In summary, this study conducted a bioinformatics analysis combined with RT-qPCR techniques to identify the highly expressed sRNA *STnc3020* in SPI-1 and within mouse macrophages. Using λ-red homologous recombination technology, we constructed *STnc3020* deletion strains, complementary strains, and strains labeled with red fluorescent protein. We systematically analyzed the impact of *STnc3020* deletion on the adhesion and invasion capabilities of *S. typhimurium* in epithelial cells, as well as its proliferation and ability to induce apoptosis in macrophages. Additionally, this study assessed the effect of *STnc3020* deletion on the virulence of *S. typhimurium.* Through various methods, including transcriptome sequencing, bacterial dual plasmid reporter systems, qRT-PCR, and Western blot, we successfully identified the target genes regulated by *STnc3020.* The findings revealed that *STnc3020* regulates the expression of the T3SS inner rod protein prgJ at the post-transcriptional level, affecting the expression levels of structural proteins and effector proteins within the T3SS secretion system, thereby maintaining the integrity and functionality of the T3SS molecular apparatus. These discoveries provide important clues and theoretical foundations for further elucidating the virulence regulation mechanisms mediated by sRNA.

Key words： *S. typhimurium*; sRNA *STnc3020*; virulence; T3SS molecular apparatus; regulatory mechanism

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