SHORT COMMUNICATION
Polish Journal of Microbiology
2023, Vol. 72, No 2, 215–219
https://doi.org/10.33073/pjm-2023-017

Transfer of T6SS$_{SPI-19}$ from *Salmonella* Gallinarum to *Salmonella* Typhimurium Lacking T6SS$_{SPI-6}$ Complements its Colonization Defect in Mice

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Submitted 23 January 2023, accepted 27 March 2023, published online 19 June 2023

Abstract

Salmonella genus harbors five Type VI Secretion System (T6SS) gene clusters. The T6SS encoded in SPI-6 (T6SS$_{SPI-6}$) contributes to *Salmonella* Typhimurium colonization of chickens and mice, while the T6SS encoded in SPI-19 (T6SS$_{SPI-19}$) of *Salmonella* Gallinarum contributes to chicken colonization. Interestingly, the T6SS$_{SPI-19}$ of *Salmonella* Gallinarum complemented the defect in chicken colonization of a *Salmonella* Typhimurium strain that lacks the T6SS$_{SPI-6}$, suggesting that both T6SSs are interchangeable. Here we show that the transfer of *Salmonella* Gallinarum T6SS$_{SPI-19}$ complemented the defect in mice colonization of a *Salmonella* Typhimurium ΔT6SS$_{SPI-6}$ strain, indicating that both T6SSs are functionally redundant during host colonization.

Keywords: *Salmonella* Typhimurium, T6SS, SPI-19, mice, colonization

The Type VI Secretion System (T6SS) is a multiprotein nanomachine made of 13 structural components and a variable number of accessory proteins that delivers bacterial proteins, called effectors, into cells through a contractile mechanism (Zoued et al. 2014; Coulthurst 2019). The extensive repertoire of effector activities makes the T6SS an efficient apparatus targeting prokaryotic and/or eukaryotic cells (Hernandez et al. 2020; Monjarás Feria and Valvano 2020), emerging as an environmental adaptation and pathogenesis factor for several bacteria (Records 2011; Basler 2015; Cianfanelli et al. 2016; Navarro-Garcia et al. 2019).

In the *Salmonella* genus, five T6SS gene clusters have been described (Blondel et al. 2009; Fookes et al. 2011; Bao et al. 2019) that belong to four distinct phylogenetic lineages (Bao et al. 2019). In addition, these gene clusters are differentially distributed among representatives of *Salmonella* (Blondel et al. 2009; Fookes et al. 2011).

The T6SS encoded in pathogenicity island SPI-6 (T6SS$_{SPI-6}$) in *Salmonella* Typhimurium (Fig. 1A) (a serotype that causes systemic infection in mice) is needed for intracellular survival within avian and murine immune cells (Parsons and Heffron 2005; Klumpp and Fuchs 2007; Mulder et al. 2012) and contributes to intestinal and systemic colonization of gavage-inoculated chickens (Pezoa et al. 2013) and mice (Mulder et al. 2012; Liu et al. 2013; Sana et al. 2016). On the other hand, current evidence indicates that the T6SS encoded in pathogenicity island SPI-19 (T6SS$_{SPI-19}$) (Fig. 1A) plays a role in gastrointestinal colonization by *Salmonella enterica* serotypes adapted to avian hosts, such as *Salmonella* Gallinarum (Blondel et al. 2010) and *Salmonella* Pullorum

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Table I
Bacterial strains and plasmids used in this study.

Strain or plasmid	Features	Source or reference
Salmonella Typhimurium		
14028s	Wild-type, virulent strain	Laboratory collection
$\Delta T6SS_{SPI-6}$	14028s Δ(*STM14_0312-STM14_0351*)::Cam	Pezoa et al. 2013
Δ*phoN*	14028s Δ(*STM14_5193*)::Kan	Pezoa et al. 2013
Δ*phoN*/R995	14028s Δ(*STM14_5193*)::Kan harboring plasmid R995	Pezoa et al. 2013
$\Delta T6SS_{SPI-6}$/R995	14028s Δ(*STM14_0312-STM14_0351*)::Cam harboring plasmid R995	Pezoa et al. 2013
$\Delta T6SS_{SPI-6}$/R995+SPI-6	14028s Δ(*STM14_0312-STM14_0351*)::Cam harboring plasmid R995+SPI-6	Pezoa et al. 2013
$\Delta T6SS_{SPI-6}$/R995+SPI-19	14028s Δ(*STM14_0312-STM14_0351*)::Cam harboring plasmid R995+SPI-19	Pezoa et al. 2013
Plasmids		
R995	Self-transmissible, broad-host range IncP plasmid	Wilson et al. 2004
R995+SPI-6	$T6SS_{SPI-6}$ gene cluster (*STM14_0312-STM14_0351*) from *Salmonella* Typhimurium 14028s cloned in plasmid R995	Pezoa et al. 2013
R995+SPI-19	$T6SS_{SPI-19}$ gene cluster (*SG1021-SG1056*) from *Salmonella* Gallinarum 287/91 cloned in plasmid R995	Blondel et al. 2010

(Xian et al. 2020). Of note, the transfer of *Salmonella* Gallinarum $T6SS_{SPI-19}$ to a $\Delta T6SS_{SPI-6}$ mutant of *Salmonella* Typhimurium complements its gastrointestinal colonization defect in chickens (Pezoa et al. 2013). Since these experiments were performed in chickens, whether $T6SS_{SPI-19}$ has a similar role in other hosts, such as mice, remains unknown.

In the present work, we evaluated the contribution of $T6SS_{SPI-19}$ to gastrointestinal colonization and systemic spread in mice using a mutant strain of *Salmonella* Typhimurium lacking the $T6SS_{SPI-6}$ and harboring the *Salmonella* Gallinarum $T6SS_{SPI-19}$ cloned in the R995 plasmid via the VEX-Capture technique (Wilson et al. 2004). Table I shows the bacterial strains and plasmids used in this work. The construction of *Salmonella* Typhimurium Δ*phoN* and $\Delta T6SS_{SPI-6}$ mutant strains has been reported (Pezoa et al. 2013). The generation of R995 plasmid derivatives harboring gene clusters $T6SS_{SPI-6}$ (39 kb) of *Salmonella* Typhimurium 14028s and $T6SS_{SPI-19}$ (42 kb) of *Salmonella* Gallinarum 287/91 has been reported (Pezoa et al. 2013; Blondel et al. 2010).

For competition experiments, groups of five BALB/c mice (6–10-week-old females) were inoculated by oral gavage with 10^6 CFU of a 1 : 1 mixture of the wild type and the mutant strain to be tested. The inoculum was calculated by serial ten-fold dilutions plated on LB agar plus suitable antibiotics. Mice were euthanized four days after infection, and the liver, spleen and cecum were collected and homogenized in sterile PBS. Titers of bacteria recovered from these organs were calculated as described above. A logarithmic mean ratio of mutant to wild-type CFU normalized to the input ratio was calculated with these numbers. Experiments involving mice were authorized by the Bioethics Committee of the School of Chemical and Pharmaceutical Sciences (Universidad de Chile) and were performed accordingly to the Guide to the Care and Use of Laboratory Animals, the Public Health Service Policy on the Human Care and Use of Laboratory Animals.

Our competition experiments showed that the *Salmonella* Typhimurium $\Delta T6SS_{SPI-6}$ strain has a defect in mice colonization, as previously reported (Mulder et al. 2012; Liu et al. 2013). The $\Delta T6SS_{SPI-6}$ mutant strain was recovered 1.3-, 2.5-, and 2.2-fold less often than the wild-type strain in the cecum, liver and spleen, respectively, at four days post-infection (Fig. 1B). The colonization defect was reversed when the mutant strain was transformed with derivatives of plasmid R995 carrying either the $T6SS_{SPI-6}$ or the $T6SS_{SPI-19}$ gene cluster from *Salmonella* Typhimurium and *Salmonella* Gallinarum, respectively (Fig. 1B), indicating that both $T6SS_{SPI-6}$ and $T6SS_{SPI-19}$ are functionally interchangeable during mice colonization. Furthermore, these results, along with those reported in the chicken model (Pezoa et al. 2013) suggest that both $T6SS_{SPI-6}$ and $T6SS_{SPI-19}$ are fully functional in distinct *Salmonella* serotypes and contribute to the colonization of different animal hosts. It is worth mentioning that the $\Delta T6SS_{SPI-6}$ mutant strain complemented with the $T6SS_{SPI-19}$ gene cluster of *Salmonella* Gallinarum colonized the spleen of infected mice more efficiently than the mutant complemented with its own $T6SS_{SPI-6}$ gene cluster (Fig. 1B). A similar trend was observed when liver colonization was evaluated. Although there is no easy explanation for this observation, it has been reported that both $T6SS_{SPI-6}$ and $T6SS_{SPI-19}$ contribute to the systemic colonization of different *Salmonella* serotypes in mice and chicken (Blondel et al. 2010; Pezoa et al. 2013; Pezoa et al. 2014). Thus, our results suggest that $T6SS_{SPI-19}$ contributes more to

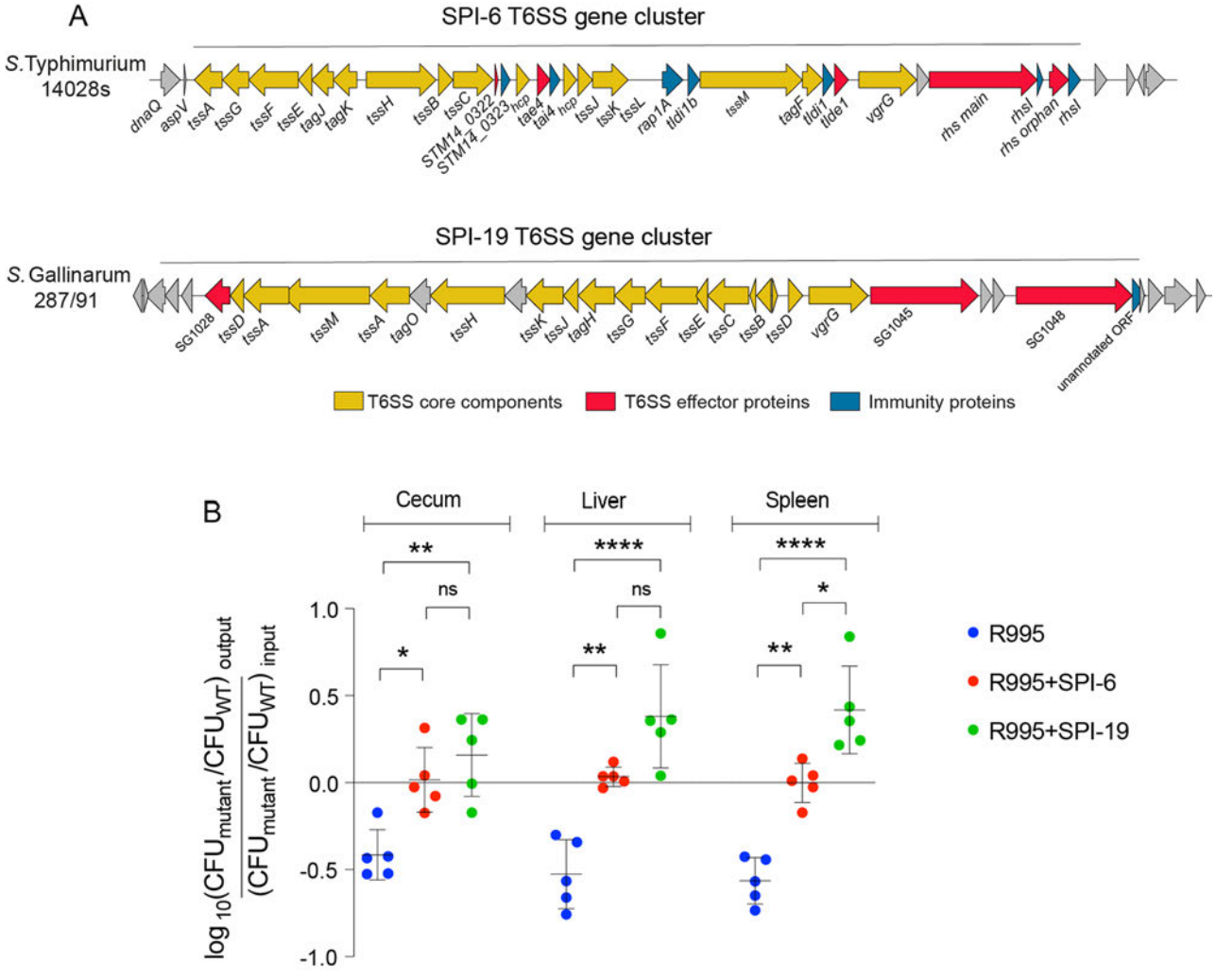

Fig. 1. *In vivo* competition between wild-type *Salmonella* Typhimurium and a ΔT6SS$_{SPI-6}$ mutant complemented *in trans* with R995, R995 + SPI6 or R995 + SPI-19.

A) Genetic organization of *Salmonella* Typhimurium T6SS$_{SPI-6}$ and *Salmonella* Gallinarum T6SS$_{SPI-19}$ gene clusters. Genes encoding core components of each T6SS are shown in yellow, and the effector and immunity protein-encoding genes are shown in red and blue, respectively. B) For competition assays, groups of six- to eight-week-old female BALB/c mice were orally infected with 10^6 CFU of a 1:1 mixture of WT/R995 with ΔT6SS$_{SPI-6}$/R995 (blue), ΔT6SS$_{SPI-6}$/R995+SPI-6 (red) or ΔT6SS$_{SPI-6}$/R995+SPI-19 (green). After four days of infection, mice were euthanized, and the cecum, liver and spleen were aseptically removed and homogenized in sterile PBS. The bacterial load recovered from each organ was determined by plating serial ten-fold dilutions on LB agar plates with appropriate antibiotics. Data show the mutant to wild-type CFU ratio normalized to the inoculum and expressed as $\log_{10}$. Bars represent mean values ± standard error. Statistical significance was determined using a one-way ANOVA test followed by Tukey's multiple comparisons tests (*$p < 0.05$; ****$p < 0.0001$; ns – not significant).

systemic colonization than T6SS$_{SPI-6}$. Further investigation is needed to elucidate this issue.

Notably, the different sets of effector proteins encoded in T6SS$_{SPI-6}$ and T6SS$_{SPI-19}$ gene clusters may play similar roles during host colonization by *Salmonella*, despite differences in resident microbiota and the immune system of mammals and birds. In this context, previous studies have characterized effector proteins with peptidoglycan hydrolase activity (Tae4, Tlde1) (Benz et al. 2013; Zhang et al. 2013; Sana et al. 2016; Sibinelli-Sousa et al. 2020; Lorente Cobo et al. 2022) and an Rhs-main toxin (Koskiniemi et al. 2014; Jurėnas et al. 2022) encoded within the T6SS$_{SPI-6}$ gene cluster that can explain the contribution of this system to animal colonization. In addition, a recent comparative genomics analysis also revealed that the *Salmonella* Gallinarum T6SS$_{SPI-19}$ gene cluster encodes two copies SG1045 and SG1048 of an effector gene homologous to SED_RS06335 of *Salmonella* Dublin (Amaya et al. 2022). SED_RS06335 is the only predicted *Salmonella* T6SS$_{SPI-19}$ effector protein with putative peptidoglycan hydrolase activity whose contribution to interbacterial competition has been experimentally demonstrated (Amaya et al. 2022). Thus, the effect of the T6SS$_{SPI-19}$ during *Salmonella* Typhimurium host colonization could be partly mediated by the antibacterial activity of these putative effectors SG1045 and SG1048 against members of the intestinal microbiota. Altogether, the presence of effector proteins in both T6SS gene clusters with peptidoglycan as a common bacterial target

site strongly suggests that $T6SS_{SPI-6}$ and $T6SS_{SPI-19}$ can be functionally interchangeable during host colonization. Besides, since a role for $T6SS_{SPI-19}$ in *Salmonella* Gallinarum interaction with immune cells has been described (Blondel et al. 2013), we cannot rule out the contribution of unidentified $T6SS_{SPI-19}$ effectors associated with this interaction during host colonization.

Altogether, we have shown that both $T6SS_{SPI-6}$ and $T6SS_{SPI-19}$ are functionally interchangeable during mice colonization, suggesting that both T6SS contribute similarly to host colonization despite having different repertoires of effector proteins. Importantly, our study highlights the utility of the VEX-Capture technique (Wilson et al. 2004) in studying the contribution of specialized protein secretion systems encoded within discrete gene clusters to bacterial pathogenesis. The main limitation of the current study is the need for more information on the contribution of specific effectors to the observed phenotypes and the molecular mechanisms involved. Further research is required to address this limitation.

Acknowledgments

DP was supported by Fondo Concursable Proyectos de Investigación Regulares UDLA 2023 DI-13/23. CAS was supported by FONDECYT grant 1212075. CJB was supported by FONDECYT grant 1201805, ECOS-ANID ECOS200037 and HHMI-Gulbenkian International Research Scholar Grant #55008749. FA was supported by CONICYT/ANID fellowship 21191925.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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