Functional Analysis of Streptococcal Virulence Factors using a Zebrafish Infection Model

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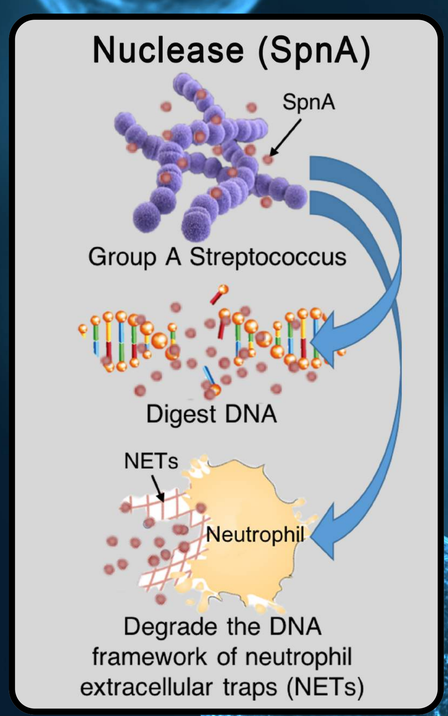
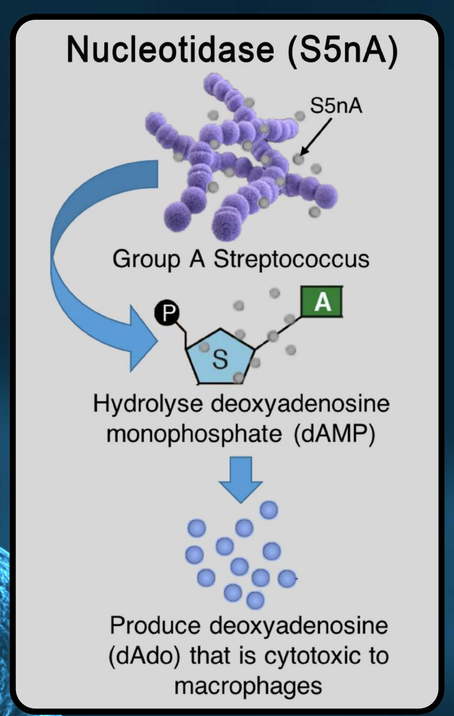
Introduction

Streptococcus pyogenes, also known as Group A Streptococcus (GAS) is an exclusively human pathogen that can cause a wide range of diseases, ranging from non-invasive pharyngitis and impetigo to more severe invasive diseases, such as toxic shock syndrome and necrotising fasciitis. We have recently characterised two novel cell wall-anchored immune evasion factors of GAS, the *Streptococcus pyogenes* nuclease A (SpnA) and Streptococcal 5'-nucleotidase A (S5nA). Investigations of GAS pathomechanisms are hindered by the lack of suitable animal infection models. We have identified two genes on the *Streptococcus iniae* genome that are similar to the GAS genes encoding SpnA and S5nA. *S. iniae* is a major fish pathogen that shares many virulence traits with GAS, and can also cause infections in humans. Biochemical analysis of the recombinant form of SpnA and S5nA from *S. iniae* (rSpnAi and rS5nAi) showed that both proteins have very similar functions *in vitro* to rSpnA and rS5nA from GAS, making them true orthologues (Soh *et al.*, 2018). In this study, we characterise the mechanisms of these two important GAS virulence factors *in vivo* by investigating their orthologues SpnAi and S5nAi using zebrafish as the infection model. These results may be used to infer the *in vivo* functions of their GAS orthologues in the human host.

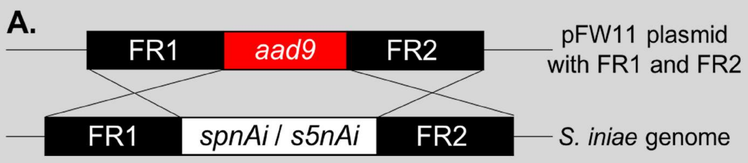




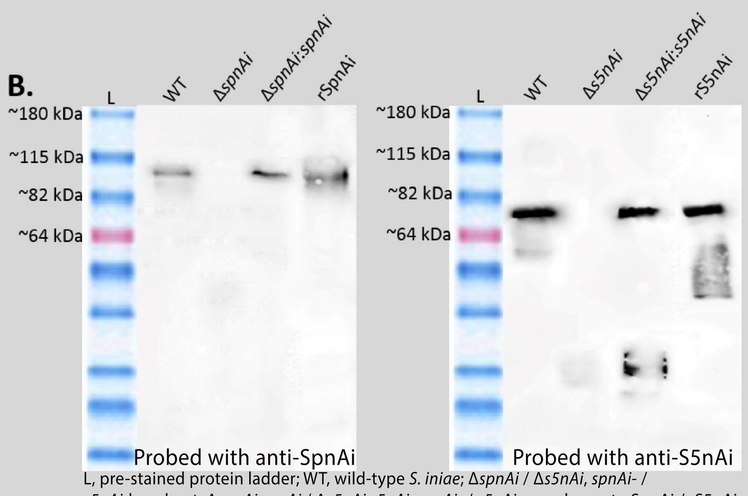
Methods and Results

Figure 1: Generation of Gene Deletion Mutants and Detection of SpnAi and S5nAi Expression by WT *S. iniae* and Mutant Strains

Gene deletion mutants (Δ*spnAi* and Δ*s5nAi*) were generated via allelic replacement of *spnAi* / *s5nAi* in *S. iniae* genome with spectinomycin resistance gene (*aad9*) on the pFW11 plasmid.



Western blot analysis of the cell wall proteins extracted from WT and mutant strains showed that SpnAi (~100 kDa) and S5nAi (~70 kDa) were expressed by WT and complementation strains, but not the Δ*spnAi* and Δ*s5nAi*.

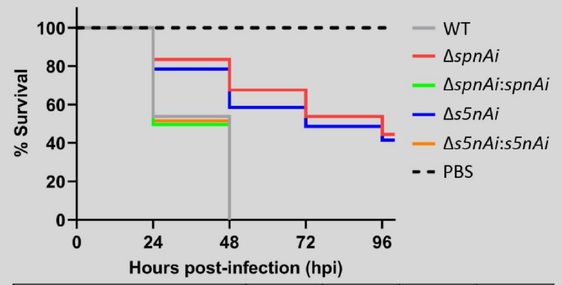


L, pre-stained protein ladder; WT, wild-type *S. iniae*; Δ*spnAi* / Δ*s5nAi*, *spnAi*- / *s5nAi*-knockout; Δ*spnAi*:*spnAi* / Δ*s5nAi*:*s5nAi*, *spnAi*- / *s5nAi*-complement; rSpnAi / rS5nAi, recombinant SpnAi / S5nAi

Figure 2: SpnAi and S5nAi contribute to *S. iniae* virulence

Survival analysis

Infection with **WT killed all larvae** within 48 hours (grey line), and infection with **Δ*spnAi* and Δ*s5nAi*** showed **survival rates of ~63% and ~60%**, respectively (red and blue lines).



Gehan-Breslow-Wilcoxon test *p*-value	Δ*spnAi*	Δ*spnAi*:*spnAi*	Δ*s5nAi*	Δ*s5nAi*:*s5nAi*
WT	****	ns	****	ns

Disease progression analysis

The **amount of bacteria** in larvae infected with **WT** (grey symbols) **increased over time** to a maximum bacteria load (**~10^5 cfu**) at 24 hpi. While, **Δ*spnAi* and Δ*s5nAi*** in larvae **increased** to a maximum bacteria load of **~10^3 cfu** at 9 hpi that was **maintained until the end** of the experiment.

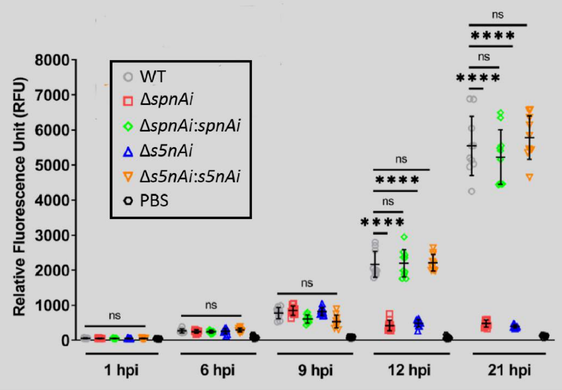
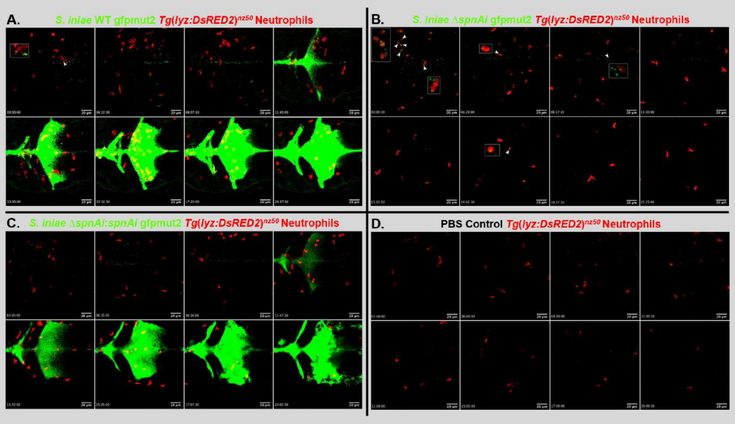


Figure 3: Analysis of Innate Immune Response to *S. iniae* Infection in Zebrafish Larvae

Host neutrophil-*S. iniae* interactions in zebrafish larvae

WT (Figure 3A) and **Δ*spnAi*:*spnAi*** (Figure 3C) were **able to escape from neutrophil killing and proliferate** at the infection site. **Deletion of *spnAi* significantly affects** the **ability of *S. iniae* to escape phagocytosis**, and to survive, proliferate and disseminate in the larvae (Figure 3B). This **confirms the role of SpnAi** as an **immune evasion virulence factor**.



Recruitment of neutrophils to the site of infection

SpnAi did not affect the **initial recruitment of neutrophils** to the infection site. However, a **significantly lower number of neutrophils recruited** to the site of infection was seen in larvae infecetd with **WT** (grey symbols) and **Δ*spnAi*:*spnAi*** (green symbols) at 14 hpi and 21 hpi. This is likely due to the **overwhelming bacterial infection** and therefore the **larval immune system** was **no longer able to cope with the bacterial infection**.

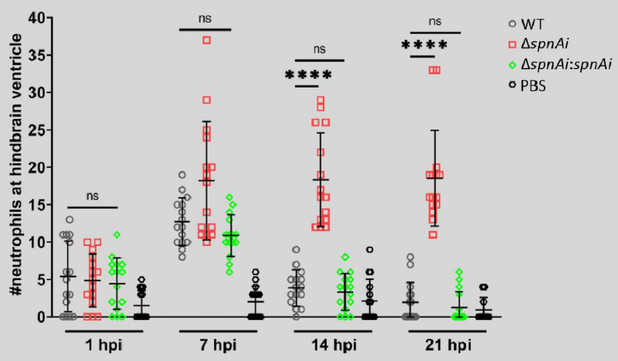
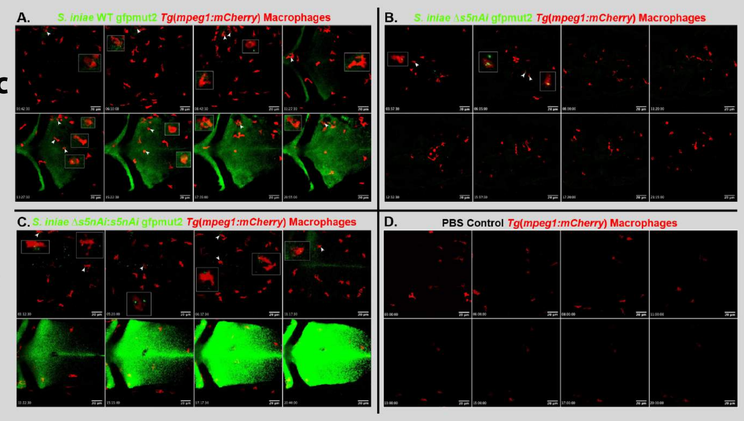


Figure 4: Analysis of Innate Immune Response to *S. iniae* Infection in Zebrafish Larvae

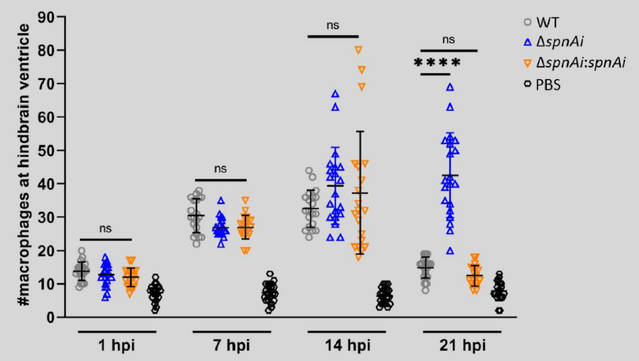
Host macrophage-*S. iniae* interactions in zebrafish larvae

WT (Figure 4A) and **Δ*s5nAi*:*s5nAi*** (Figure 4C) were able to **suppress the phagocytic function of macrophages**, thereby **promoting bacterial survival and proliferation** in larvae. **Deletion of *s5nAi* significantly affects** the **ability of *S. iniae* to survive**, proliferate and disseminate in the larva (Figure 4B). This **confirms the role of S5nAi** as an **immune evasion virulence factor**.

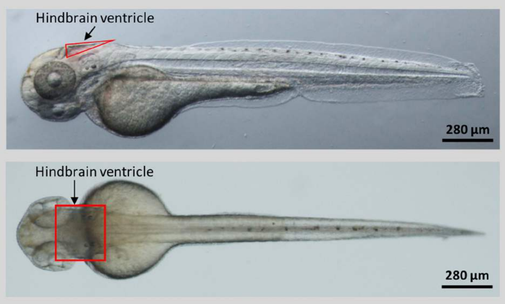


Recruitment of macrophages to the site of infection

S5nAi did not affect the **initial recruitment of macrophages** to the infection site. The **low number of macrophages** at the hindbrain ventricle of larvae infected with **WT** (grey symbols) and **Δ*s5nAi*:*s5nAi*** (orange symbols) at 21 hpi was **possibly due to the production of high amount of deoxyadenosine** that **activate macrophage caspase-3-mediated apoptosis**.



Zebrafish Larvae



Two days post-fertilisation zebrafish larvae in lateral view (top) and dorsal view (bottom). Black arrow indicates the site of infection at hindbrain ventricle. Confocal live cell imaging of the area surrounded by the red square was performed.

Conclusion

- **SpnAi and S5nAi activities contribute to *S. iniae* virulence.**
- **SpnAi and S5nAi promote bacterial survival in zebrafish larvae.**
- **SpnAi and S5nAi did not affect the recruitment of neutrophils and macrophages to the infection site, respectively. However, the neutrophil and macrophage phagocytic functions were affected.**
- ***S. iniae* zebrafish-larvae infection model was successfully established and maybe used to investigate other streptococcal virulence factors.**

Scan the QR code below using your mobile phone to view video of confocal live cell imaging

Figure 3 - Neutrophil responses to *S. iniae* infection

Figure 4 - Macrophage responses to *S. iniae* infection

Reference:
Soh, K. Y., Loh, J. M. S., & Proft, T. (2018). Orthologues of *Streptococcus pyogenes* nuclease A (SpnA) and Streptococcal 5'-nucleotidase A (S5nA) found in *Streptococcus iniae*. *The Journal of Biochemistry*, 164(2), 165-171.