Phagosomal escape of hypervirulent *Staphylococcus aureus* is not dependent on beta-toxin expression

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Introduction and Objectives

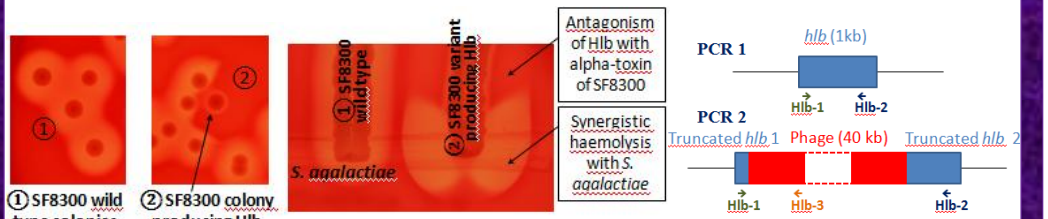
Intracellular virulence of *S. aureus* is of recent description and its underlying mechanisms are still debated. Death induction in *S. aureus*-infected host cells reportedly requires **phagosomal escape** of the bacteria, which is shown to **depend on beta-toxin (Hlb)** expression in laboratory strains (1).

Most **clinical strains** however, including hypervirulent strains such as community-acquired methicillin-resistant *S. aureus* (CA-MRSA) USA300, **do not produce Hlb** because of the insertion of a prophage in the *hlb* gene - yet these strains are able to escape phagosomes.

To resolve this apparent contradiction, several authors have proposed that intra-phagosomal oxidative stress induces the **excision of the converting phage**, thus restoring Hlb expression in the phagosomal compartment (2). Here we tested this hypothesis using both *in vitro* and *ex vivo* cell culture models.

Materials and Methods

We developed a high-throughput phenotypic method for **detecting functional Hlb expression** based on haemolysis profile analysis on sheep blood agar. Method specificity was confirmed both phenotypically using a) a modified CAMP-test (synergistic haemolysis with *Streptococcus agalactiae*) and genotypically using b) PCR to assess phage excision and the restoration of the native *hlb* gene.



a) Modified CAMP-test b) PCR reactions used for confirmation

The **rates of phage excision** were then quantified in the hypervirulent CA-MRSA prototype strain USA300-SF8300 in experiments of *in vitro* **oxidative stress** (3h incubation with or without 0.9 mM H_2O_2) and of *ex vivo* **intracellular passage** (6h intracellular infection of MG-63 osteoblastic cells in a gentamicin protection assay at MOI 100:1, or 6h incubation in DMEM cell culture medium without MG-63 cells). Approximately 1000 bacteria were observed per condition in 2 independent experiments.

Finally, the cytotoxicity of Hlb-positive and -negative USA300-SF8300 isolates was measured using an LDH release assay after 24h intracellular infection of MG-63 cells in 2 independent experiments.

References

(1) Giese B, Glowinski F, Paprotka K et al. *Cell Microbiol* 2011.
(2) Fraunholz M and Sinha B. *Front Cell Infect Microbiol* 2012.

Results

In *in vitro* experiments, *hlb*-converting phage excision occurred with a low but non-neglectable basal rate of 0.03%, which was increased 15.3-fold after H_2O_2 exposure (0.46%; $p < 0.01$, Fisher's exact test). In *ex vivo* experiments however, the rate of phage excision was not increased after intracellular passage as compared to bacteria incubated in eukaryotic cell-free medium (0.11 vs. 0.12%, respectively; $p = 1.00$).

Moreover, no difference was found between Hlb-positive and -negative isolates with respect to MG-63 cell death induction ($p = 0.27$).

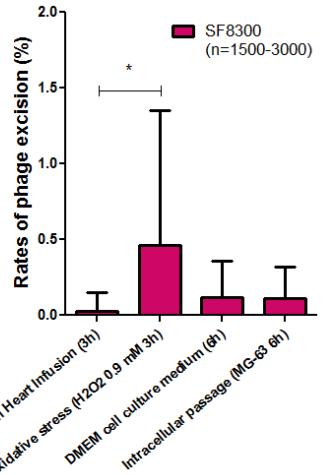


Figure 1. Rates of phage excision in SF8300 strain dependent on culture conditions. n: number of colonies observed. Fisher's test : *$p < 0.05$.

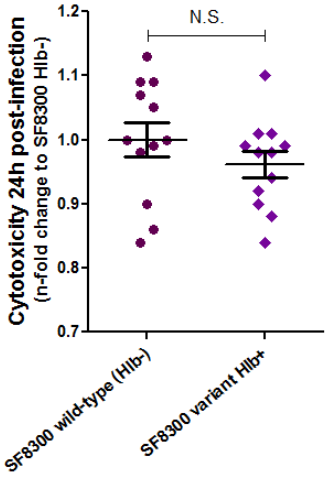


Figure 2. Impact of Hlb production on the cytotoxicity of SF8300 strain. t-test; N.S.: not significant.

Conclusions

The **excision** of the *hlb*-converting phage occurs **spontaneously** at a low frequency; although phage excision is significantly induced by oxidative stress *in vitro*, it is neither induced nor selected after intracellular passage, nor is it associated with an increased cytotoxicity.

Collectively, these findings **rule out a role of Hlb** in the **intracellular virulence of hypervirulent CA-MRSA**. Alternative phagosomal escape mechanisms likely exist in these strains, and future research efforts should focus on the role of other exotoxins such as phenol-soluble modulins.