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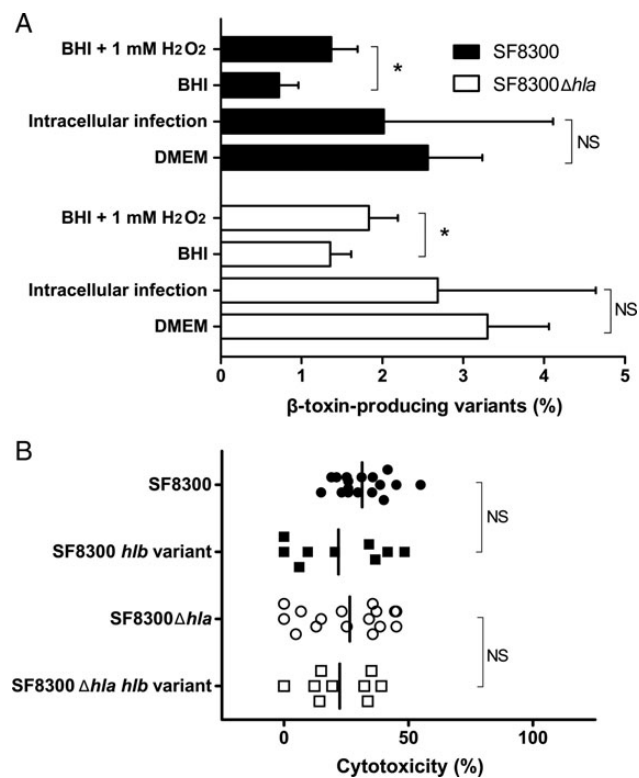
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# Correspondence

## Does $\beta$ -toxin Production Contribute to the Cytotoxicity of Hypervirulent *Staphylococcus aureus*?

TO THE EDITOR—The recent article by Salgado-Pabón et al, which reported that excision of the staphylococcal  $\beta$ -toxin-converting bacteriophage  $\phi$ Sa3 is common in  $\phi$ Sa3-positive strains, was interesting [1]. By demonstrating that  $\beta$ -toxin production is selected for by in vivo infection and enhances virulence in rabbit models of pneumonia and infective endocarditis, Salgado-Pabón et al identified a previously unsuspected role for  $\beta$ -toxin in  $\phi$ Sa3-positive *Staphylococcus aureus*. Regarding infective endocarditis, they insightfully predicted that the increase in vegetation size associated with  $\beta$ -toxin production could be linked to its nucleoprotein ligase activity. However, other mechanisms by which  $\beta$ -toxin could contribute to pathogenesis in these models were not discussed. Interestingly,  $\beta$ -toxin has been shown to participate in the intracellular virulence of *S. aureus* [2].  $\beta$ -toxin production, along with production of phenol-soluble modulins, is reportedly required for the disruption of phagosomal membranes by *S. aureus* after internalization and for the bacterium to gain access to the cytoplasm of the infected cell, eventually triggering cell death. Interestingly, hypervirulent strains such as *S. aureus* USA300 escape phagosomes and are highly cytotoxic despite harboring  $\phi$ Sa3 [3]. Facing this apparent contradiction, some authors hypothesized that intraphagosomal oxidative stress induces  $\phi$ Sa3 excision and restores  $\beta$ -toxin production [4]. The finding that  $\phi$ Sa3 excision is common during in vivo infection adds credibility to this hypothesis, which we have investigated recently.

We used the hemolysis profile analysis on sheep blood agar plates described by Salgado-Pabón et al to quantify the frequency of  $\beta$ -toxin variants in *S. aureus* populations. Functional  $\beta$ -toxin production was confirmed using a CAMP test,



**Figure 1.** The excision of the  $\beta$ -toxin-converting phage  $\phi$ Sa3 is induced by oxidative stress but not by intracellular infection and does not enhance cytotoxicity in the hypervirulent *S. aureus* strain SF8300. **A**, The proportion of  $\beta$ -toxin-producing variants was significantly higher in strains SF8300 and SF8300 $\Delta$ hla after 3 hours of incubation in brain-heart infusion (BHI) broth with 1 mM H<sub>2</sub>O<sub>2</sub>, compared with BHI broth alone; however, the same result was not observed after 6 hours of intracellular infection of MG-63 osteoblastic cells, compared with Dulbecco's modified Eagle's medium (DMEM) alone. The results were obtained from 3 independent experiments. Approximately 300 colonies were analyzed after intracellular passage, and 3000 colonies were analyzed under the other conditions. Differences in proportions were tested for significance, using the Fisher exact test. Error bars indicate exact binomial 95% confidence intervals. **B**,  $\beta$ -toxin-producing variants of strains SF8300 and SF8300 $\Delta$ hla did not exhibit enhanced cytotoxicity in the intracellular infection model of MG-63 cells with a 24-hour incubation period. The cytotoxicity percentage was quantified using a lactate dehydrogenase-based assay, with uninfected cells and cells lysed by osmotic shock corresponding to 0% and 100% toxicity, respectively. The results were obtained from 3 independent experiments performed in triplicate. Differences between groups were tested using the Mann-Whitney *U* test. The significance threshold was set at .05 for all tests. \**P* < .05. Abbreviations: *hla*,  $\alpha$ -toxin-encoding gene; *hnb*,  $\beta$ -toxin-encoding gene; NS, not significant.

and polymerase chain reaction analysis was performed to assess phage excision and the restoration of the native  $\beta$ -toxin gene, as described elsewhere [5]. We first determined whether  $\phi$ Sa3 excision was induced under oxidative stress conditions in vitro. Incubation of the hypervirulent *S. aureus* strain USA300-SF8300 for 3 hours in brain-heart infusion (BHI) broth containing H<sub>2</sub>O<sub>2</sub> at the phage-inducing concentration of 1 mM [6] moderately increased the frequency of  $\beta$ -toxin-producing variants, compared with incubation in BHI broth alone (Figure 1A). As indicated by Salgado-Pabón et al, detection of  $\beta$ -toxin-producing variants can be difficult in the presence of  $\alpha$ -toxin. We thus reproduced the experiments with an  $\alpha$ -toxin-negative ( $\Delta hla$ ) mutant of strain SF8300 (kindly provided by B. A. Diep) and observed the same pattern (Figure 1A). Of note, the basal rate of  $\phi$ Sa3 excision in strains SF8300 and SF8300 $\Delta hla$  was approximately 1%, which is consistent with the rates reported in strain MW2 [1].

We then investigated whether  $\phi$ Sa3 excision was induced after the intracellular infection of eukaryotic cells by *S. aureus*. We used a well-established in vitro model of intracellular infection of MG-63 human osteoblastic cells, with a multiplicity of infection of 100 [3]. In this model, the proportion of  $\beta$ -toxin-producing variants in SF8300 and SF8300 $\Delta hla$  recovered after 6 hours of intracellular infection (ie,  $\geq 3$  hours of intracellular localization, after subtracting 2 hours of coculture and 1 hour of gentamicin-based selection of intracellular bacteria [3]) did not differ significantly from that found in bacteria incubated for the same amount of time in cell culture medium alone. These findings suggest that intracellular infection does not induce  $\phi$ Sa3 excision.

Finally, we determined whether the ability of strains SF8300 and SF8300 $\Delta hla$  to induce infected MG-63 cell death was influenced by  $\beta$ -toxin production. Spontaneous  $\beta$ -toxin-producing variants of SF8300 and SF8300 $\Delta hla$  (3 randomly selected variants per strain) were used

along with the parental strains to infect MG-63 cells. Intracellular infection was prolonged for 24 hours, after which cytotoxicity was assessed using a lactate dehydrogenase-based assay. The cytotoxicity of the  $\beta$ -toxin-producing variants did not differ from that of the wild-type strain in both SF8300 and SF8300 $\Delta hla$  (Figure 1B), which is evidence against the contribution of  $\beta$ -toxin to intracellular virulence in this model.

Overall, these data do not support either the induction or involvement of  $\beta$ -toxin during the intracellular infection of MG-63 cells by *S. aureus* USA300-SF8300 [3]. Hence, the contribution of  $\beta$ -toxin to  $\phi$ Sa3-positive *S. aureus* pathogenesis in vivo, which was firmly established by Salgado-Pabón et al, is not likely related to host cell invasion. The consequences of this observation are 2-fold. First, our findings suggest that  $\beta$ -toxin-related pathogenesis occurs in the extracellular compartment, which is in agreement with the mechanisms proposed by Salgado-Pabón et al [1]. Second, these results indicate that the cytotoxicity of hypervirulent *S. aureus* does not require  $\beta$ -toxin production. Further studies are warranted to determine how  $\beta$ -toxin contributes to *S. aureus* pathogenesis in vivo and to decipher the mechanisms by which hypervirulent *S. aureus* kills infected cells in a  $\beta$ -toxin-independent fashion.

## Notes

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