Use of a *Staphylococcus aureus* Small Colony Variant as an Attenuated Live Vaccine for the Prevention of Intramammary Infections

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Introduction

Staphylococcus aureus is a major human and animal pathogen that can cause high morbidity, severe infections as well as difficult-to-treat, chronic forms of diseases. Furthermore, incidences of *S. aureus* infections are becoming more worrisome with the emergence of multiple antibiotic resistant strains. In the case of bovine mastitis, a major problem for the dairy industry, *S. aureus* is the most frequently isolated contagious pathogen. The development of vaccines for the prevention and control of *S. aureus* Intramammary infections (IMIs) is an interesting alternative although no formulation has demonstrated protective efficacy to date, possibly because of the use of inadequate vaccine targets or the failure to elicit an appropriate immune response.

Background

In a previous study, we used transcriptional analysis to uncover *S. aureus* genes that were highly expressed by several strains in an experimentally induced bovine IMI[1]. One gene was shown to be a good target for a new drug therapy[2], and **SACOL0720** (*vraG*), recently associated with antimicrobial peptide sensing and resistance, was proven to be important in virulence by the strong attenuation of its **Δ720** inactivation mutant in an experimental IMI[1].

The use of attenuated live bacteria to deliver antigens should help developing a more complete humoral and cellular immune response. Small colony variants (SCVs) of *S. aureus* are characterized by a dysfunctional oxidative metabolism, causing a slower growth and alteration in the expression of virulence factors. SCVs also are easily internalized in host cells and persist without producing invasive (destructive) infections[3]. Besides, laboratory deletion of gene ***hemB*** generates a stable SCV unable to revert to the normal phenotype.

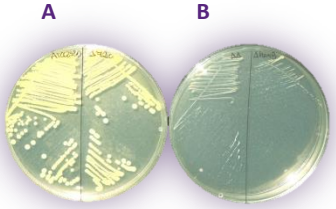

Fig. 1. Aspects of normal (A) and SCV (B) phenotypes on TSA plates (48h)

Objectives:

- Engineer a stable *S. aureus* SCV by deletion of gene *hem*B (Δ*hem*B)
- Further attenuate Δ*hem*B through inactivation of SACOL0720 to obtain Δ*hem*BΔ720 for future use as a live vaccine vector
- Evaluate persistence and invasion of the Δ720Δ*hem*B vaccine strain in a mammalian cell infection assay
- Attest attenuation of Δ*hem*BΔ720 in a murine IMI model

Materials and Methods

- The Δ720, Δ*hemB* and double Δ*hemB*Δ720 mutants were obtained in *S. aureus* ATCC 29213. Δ720 was generated using the TargeTron (Sigma) gene knockout system by insertion of a group II intron in SACOL0720. The deletion of gene *hemB* was achieved by gene replacement with an *emrA* cassette in parental and Δ720 strains. Mutations were confirmed by phenotypic characterization and PCR.
- All four strains were assayed for persistence in the bovine mammary gland epithelial cells (BMEC) line MAC-T. After a 3-h incubation of cells with bacteria at a MOI of 10, cells were incubated in presence of lysostaphin to kill extracellular bacteria for an additional 12h or 24h before cells were washed and lysed for intracellular bacterial counts.
- The virulence of the Δ*hemB*Δ720 mutant was assayed and compared to the parental strain ATCC 29213(WT) in a murine IMI model. CD-1 lactating mice were inoculated under anesthesia by direct injection through the teat canal of the large R4 and L4 mammary glands using 100 CFU of the double mutant or parental strain. CFUs per gram of harvested glands were evaluated after 6h, 12h and 1, 2, 4, 7 and 12 days of infection.

Results: BMEC Invasion Studies

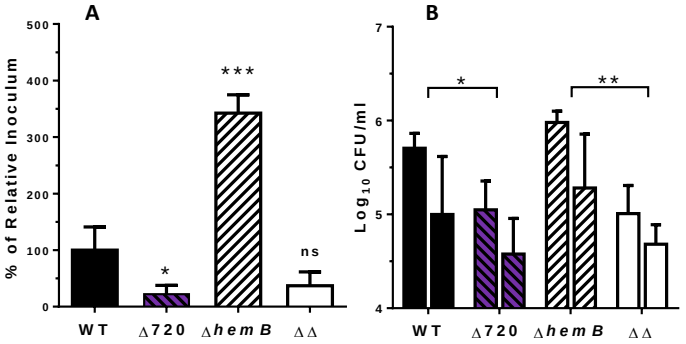

Fig. 2. Intracellular CFU counts of parental (WT) and mutant strains. MAC-T cells were infected with the four strains for 3h, then were incubated with lysostaphin an additional 12 or 24h and lysed. (A) Relative percentage of the initial inoculum found within cells for each mutant compared to that obtained for parental strain (WT; 100%) at 12h. Standard deviations (SD) and statistical significance are compared to the corresponding WT (One-way ANOVA; Dunnett's multiple comparisons test). (B) Means and SD of bacterial CFU counts at 12h (left bar) and 24h (right bar). (Two-way ANOVA and Tukey's multiple comparisons test, *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$).

- The double mutant (ΔΔ) shows a **reduced intracellular count** but persists in cells like Δ*hemB* **without provoking cell destruction** like the wild type.
- **Gene 0720 is important for intracellular survival** and **a good attenuation target** for both normal (WT) and SCV (Δ*hemB*) phenotypes as Δ720 strains showed **significantly lower CFUs** compared to their isogenic strains in this mammalian cell infection model.

Results: Mouse IMI *in vivo* Studies

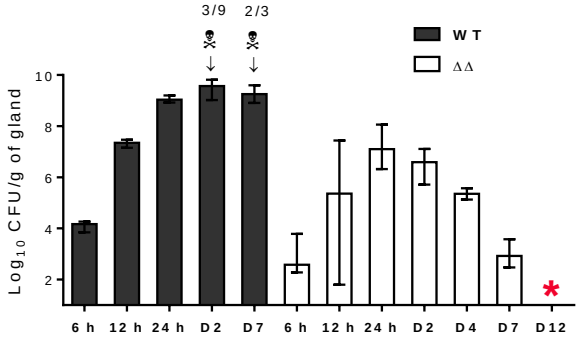

Fig. 3. Murine IMIs with the parental (WT) and Δ*hemB*Δ720 (ΔΔ) strains over time. Mice were infected and glands harvested at the indicated hour (h) or day (D) after infection. Each column represents the median value of bacterial CFU counts for a group of glands, and ranges are indicated by bars. Six glands/group were used excepted for wild type strain at D7 (only one mouse survived). Mortality is indicated by arrows. The red star indicates bacterial clearance for Δ*hemB*Δ720.

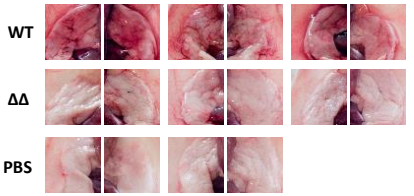

Fig. 4. Inflammation of the large R4 and L4 mammary glands 24 h after infection. Glands were visually scored for inflammation and pictures were taken for comparison.

- **The ΔΔ mutant was strongly attenuated** compared to its parental strain. The bacterial CFUs for **ΔΔ were reduced by 5 log10** at day 7 and **bacterial clearance achieved at day 12**. Inflammation was also reduced after 24 h.
- **The parental strain provoked severe infections**, killing 5 of 12 mice. Surviving mice maintained high viable counts (≥9 $\log_{10}$ CFUs/gland).

Conclusions

We have successfully obtained an attenuated *S. aureus* strain. This Δ720Δ*hemB* mutant is a stable SCV that could be used for intracellular delivery of *S. aureus* antigens. Such a live vaccine vector could enhance cell-mediated immunity against *S. aureus* IMIs.

References

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2. Ster *et al.* 2013. Experimental treatment of *Staphylococcus aureus* bovine intramammary infection using a guanine riboswitch ligand analog. J. Dairy Sci. 96(2):1000-8
3. Brouillette *et al.* 2004. Persistence of *Staphylococcus aureus* small-colony variant under antibiotic pressure *in vivo*. FEMS Immunol. Med. Microbiol. 41(1):35-41