

SHORT REPORT



## O-Acetylation is essential for functional antibody generation against *Staphylococcus aureus* capsular polysaccharide

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### ABSTRACT

*Staphylococcus aureus* produces an antiphagocytic polysaccharide capsule to evade neutrophil-mediated killing. Many vaccines against encapsulated bacterial pathogens require generation of functional anti-capsular antibodies to mediate protection against infection and disease. Here it is shown that the generation of such antibody responses to *S. aureus* *in vivo* and *in vitro* requires the presence of O-acetyl modifications on the capsular polysaccharides. O-acetylation of *S. aureus* capsular polysaccharide therefore should be monitored carefully during vaccine development and production. This finding may provide additional insight into the previous failure of a *S. aureus* capsular polysaccharide conjugate vaccine.

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### Introduction

The medical need for a *Staphylococcus aureus* vaccine is clear. Although *S. aureus* is carried asymptotically in the nares of 20–50% of the general population,<sup>1</sup> colonization increases the risk of infection, ranging from relatively mild skin infections, such as impetigo, to life-threatening invasive disease. *S. aureus* is recognized as a leading cause of morbidity and mortality in both healthcare-associated and community settings. In particular in surgical patients, these infections carry high mortality rates and survivors of *S. aureus* surgical infections require an additional 13–17 days in the hospital, significantly increasing healthcare costs.<sup>2</sup> The burden of *S. aureus* disease is exacerbated by the emergence of *S. aureus* isolates that are resistant to new classes of antibiotics, highlighting the need for alternative approaches such as a prophylactic vaccine.

The human host employs several mechanisms to prevent *S. aureus* infection. These include mucosal and epithelial layers that act as a barrier to infection, and engulfment and killing of bacteria by professional phagocytes, such as neutrophils. However, *S. aureus* exploits virulence mechanisms that counteract such host defenses, thus facilitating establishment of an infection. These virulence mechanisms include scavenging of essential nutrients from blood, adhesion to host tissues and immune evasion.<sup>3, 4</sup> Therefore, a successful vaccine will need to neutralize such virulence mechanisms.

Capsular polysaccharides help bacteria evade immune-mediated killing through inhibiting phagocytosis.<sup>5, 6</sup> Vaccine-induced antibodies against capsular polysaccharides can overcome this virulence mechanism by enabling the organism to be opsonized and subsequently phagocytosed. All invasive human *S. aureus* isolates encode the genes required to express either type 5 or type 8 capsule (denoted CP5 and CP8, respectively),<sup>7</sup> and most adults have anticapsular antibodies, demonstrating

that the capsule is expressed in the host environment.<sup>8</sup> Bacterial polysaccharides often contain an array of substituents, such as O-acetyl, phosphate, sialic acid, which may constitute an important part of the immunodominant epitopes.

Both *S. aureus* CP5 and CP8 are comprised of 2-acetamido-2-deoxy-D-mannuronic acid (ManNAcA), 2-acetamido-2-deoxy-L-fucose (L-FucNAc), and 2-acetamido-2-deoxy-D-fucose. Both capsules are O-acetylated and differ in the stereochemical glycosidic linkages between the sugars and the site of O-acetylation. The sites of O-acetylation are the 3'OH moiety of L-FucNAc for CP5 and 4'OH substituent of ManNAcA for CP5.<sup>9</sup> Here, it is shown that O-acetylation of capsular polysaccharides is critical for the induction of protective anticapsular antibody responses upon vaccination.

### Materials and methods

#### Production of de-O-Acetylated *S. aureus* capsular polysaccharide conjugates

CP8 and CP5 were isolated as previously described<sup>10</sup> from CP5 expressing strain BD285 and CP8 expressing strain BD287, then de-O-acetylated by incubation in 0.2 N NaOH or 0.2 N TBOH, respectively (Sigma) at 37°C for 4 h. Samples were neutralized with HCl, washed with water, and resuspended at ~4 mg/mL as determined with 4-hydroxybenzoic acid hydrazide (PAHBAH). Removal of O-acetyl groups was verified by size exclusion chromatography multi angle laser light scattering (SEC MALLS). De-O-acetylated CP5 and CP8 and native CP5 were conjugated to diphtheria toxin mutant CRM<sub>197</sub> via reductive amidation.

#### Opsonophagocytic activity assay (OPA)

OPAs were performed as previously described.<sup>5</sup> Baby rabbit complement (BRC) was used as a source of complement in the

assay containing bacteria (either CP5-expressing *S. aureus* strain PFESA0158 or CP8-expressing *S. aureus* strain PFESA0186, both clinical isolates), serially diluted heat-treated serum and HL-60 cells (Pel-Freez, Cat. #: 31061 3). An OPA antibody titer was defined as the reciprocal of the highest serum dilution resulting in 50% reduction of the number of bacterial colonies after incubation for 60 min at 37°C when compared to the background control from which serum was omitted. Samples without detectable activity at the lowest serum dilution of 100 were assigned OPA titer values of 50.

### Murine pyelonephritis model

All animal studies were conducted under the guidance of the local and global institutional animal care and use committee at an AAALAC-accredited facility. Female CD-1 mice (20/group) were immunized three times subcutaneously on weeks 0, 3 and 6 with 1 µg of de-O-acetylated CP5-CRM<sub>197</sub> + AlPO<sub>4</sub>, de-O-acetylated CP8-CRM<sub>197</sub> + AlPO<sub>4</sub>, native CP5-CRM<sub>197</sub> + AlPO<sub>4</sub>, or vehicle alone (AlPO<sub>4</sub>). Two weeks after the final immunization, mice were challenged with ~3 × 10<sup>8</sup> CFU *S. aureus* Reynolds. Kidneys were harvested 48 h after infection, homogenized, and serial dilutions plated on tryptic soy agar. Bacterial colonies were enumerated after 24 h of incubation at 37°C.

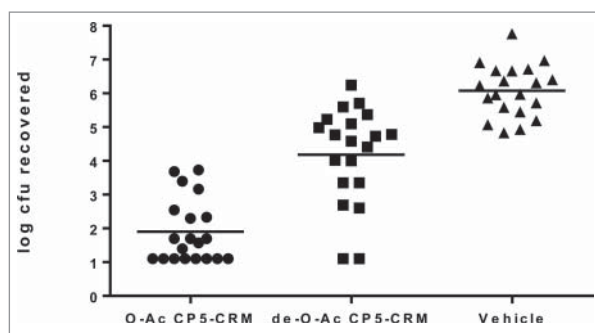
### Statistical analysis

All statistical comparisons were performed using unpaired Student's t test with Welch's Correction by GraphPad Prism software v7.02.

### Results and discussion

The presence of O-acetyl groups in capsular polysaccharides has been observed in a wide range of pathogenic bacteria, including *N. meningitidis* serogroup A and *S. pneumoniae* 9V. However, the role of O-acetylation in the immunogenicity and pathogenicity of microorganisms cannot be generalized. While capsular polysaccharide O-acetylation induces functional responses against *N. meningitidis* serogroup A,<sup>11</sup> for *S. pneumoniae* 9V<sup>12</sup> the presence of O-acetyl groups mask epitopes important for protective immune responses, thus misdirecting the immune response and allowing bacterial immune evasion.<sup>13</sup>

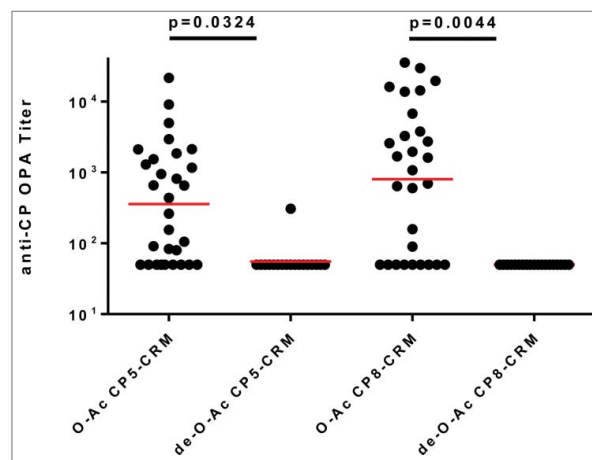
To determine if de-O-acetylated CP5-CRM<sub>197</sub> elicited a protective immune response after immunization, a pyelonephritis model of disseminated end-organ infection was used. Female CD-1 mice were immunized three times with O-acetylated (OAc) or de-O-acetylated (deOAc) CP5-CRM<sub>197</sub> adjuvanted with AlPO<sub>4</sub>, or AlPO<sub>4</sub>-containing vehicle alone, and subsequently challenged with *S. aureus*. Use of vehicle resulted in a high kidney burden of 10<sup>6</sup> CFU. Although immunization with de-O-acetylated CP5-CRM<sub>197</sub> reduced *S. aureus* burden in the kidneys compared to vehicle alone, O-acetylated CP5-CRM<sub>197</sub> reduced kidney bacterial burden by over 2 logs more than the de-O-acetylated CP5-CRM<sub>197</sub>, a statistically significant decrease (Figure 1,  $p < 0.0001$ ). To test whether O-acetylation of *S. aureus* capsular polysaccharides impacts generation of



**Figure 1.** O-acetylation is necessary for an effective protective vaccine response in a murine pyelonephritis model. Female CD-1 mice (20/group) were immunized three times with 0.01 mcg of O-acetylated (native) CP5-CRM<sub>197</sub> with AlPO<sub>4</sub>, de-O-acetylated CP5-CRM<sub>197</sub> with AlPO<sub>4</sub>, or AlPO<sub>4</sub> alone, then challenged with *S. aureus*. Bacterial burden in the kidneys was determined two days after challenge.

functional immune responses, it was first established that a dose of 0.01 mcg of capsular polysaccharide conjugate was the lowest threshold dose level able to induce opsonophagocytic antibodies (data not shown). The use of a low threshold dose allowed for the sensitive discrimination of differences in elicitation of opsonophagocytic antibodies. Female CD-1 mice were immunized three times with either native or de-O-acetylated CP5-CRM<sub>197</sub> or native or de-O-acetylated CP8-CRM<sub>197</sub>, each in the presence of AlPO<sub>4</sub>. Sera were collected one week after the last immunization and assessed for opsonophagocytic activity (OPA, Figure 2). Neither de-O-acetylated CP5-CRM<sub>197</sub> nor de-O-acetylated CP8-CRM<sub>197</sub> were able to elicit detectable OPA responses in all but one animal, while their native counterparts elicited OPA responses in the majority of animals (Figure 2). This result indicates that immunization with the de-O-acetylated polysaccharide was unable to protect in vivo due to the inability to induce functional antibodies that resulted in opsonophagocytic killing.

*S. aureus* CP-CRM<sub>197</sub> conjugates have previously been shown to induce robust bacterial killing responses as measured in OPA (5, Figure 2) and capsular polysaccharide is known to be an important immune evasion mechanism for *S. aureus*.



**Figure 2.** O-acetylation is necessary for capsular polysaccharide conjugates to induce effective opsonophagocytic killing responses. Female CD-1 mice (20/group) were immunized three times with 0.01 mcg of O-acetylated (native) CP5-CRM<sub>197</sub> with AlPO<sub>4</sub>, de-O-acetylated CP5-CRM<sub>197</sub> with AlPO<sub>4</sub>, O-acetylated (native) CP8-CRM<sub>197</sub> with AlPO<sub>4</sub>, or de-O-acetylated CP8-CRM<sub>197</sub> with AlPO<sub>4</sub> and OPA titers from week 7 sera were assessed.

Studies by Fattom et al.<sup>14</sup> suggested that antibodies to both OAc and non-OAc epitopes of *S. aureus* CP5 and CP8 opsonized O-acetylated isolates are functional and efficient against variations in O-acetylation of capsules polysaccharides expressed by clinical isolates. In contrast, here we have demonstrated that O-acetylation is required to induce effective functional killing responses to *S. aureus* as measured both *in vitro* by OPA killing assay, and *in vivo* by protection in the murine pyelonephritis model. Although there were experimental differences between the studies, such as the carrier protein used for the conjugate (CRM<sub>197</sub> vs *Pseudomonas aeruginosa* exotoxin A), and the details of the opsonophagocytic assays used, which could have contributed to the divergent observations in the studies, it should also be noted that the Fattom et al study conducted the OPAs with highly purified rabbit IgG. *In vivo*, immunization with non-O-acetylated CP5 did reduce recovered CFU compared to vehicle alone (Figure 1); likewise the Fattom et al study could have been observing this minor effect using highly purified rabbit IgG. Using a competitive luminex immunoassay, which monitors the ability of immunization-induced antibodies to compete with a functional monoclonal antibody, serum from animals immunized with de-O-acetylated CP5 and CP8 conjugates had very low titers compared to animals immunized with O-acetylated CP5 and CP8 conjugates (data not shown). Together, these results support the hypothesis that de-O-acetylated conjugates are poor functional immunogens.

A tetra-antigen *S. aureus* vaccine (SA4Ag) is currently in development, which contains capsular polysaccharide types 5 and 8 conjugated to CRM<sub>197</sub> as well as clumping factor A (ClfA) and the manganese transport component MntC. Pivotal vaccine trials with an earlier capsular polysaccharide conjugate vaccine were not successful. While this capsular polysaccharide conjugate vaccine did show a 57% decrease in bacteremia versus controls in hemodialysis patients, no significant protection was observed in a larger phase 3 trial.<sup>15</sup> These results sharply contrast the striking successes of capsular polysaccharide-based vaccines in protecting against disease caused by *Hemophilus influenzae* type B (HiB), *Neisseria meningitidis* types A, C, Y and W and *Streptococcus pneumoniae*. The reasons for this failure have been speculated about. One factor is that there may have been issues with consistency of manufacture of the phase 3 clinical trial material, due to changes in contract facilities used, although the specific impacts of these changes were not further explained.<sup>15</sup> It is also possible that the assays used to monitor vaccine quality were not sufficiently sensitive to detect important critical quality attributes. Lack of access to the material used in the phase 3 study precludes a direct comparison between earlier results and the data presented here. The data presented here indicates that O-acetylation of the *S. aureus* capsular polysaccharide is critical for vaccine-induced functional immune responses *in vitro* and *in vivo*, and variations therein should be minimized to ensure consistent functional immune responses and efficacy.

### Disclosure of potential conflicts of interest

All authors are employees of Pfizer, Inc., and as such may hold stock and/or stock options. This work was presented at the *Staphylococcus aureus* Gordon Conference (July 2013, Waterville Valley, NH).

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