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Biologic properties and vaccine potential of the staphylococcal poly-*N*-acetyl glucosamine surface polysaccharide

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Abstract

Staphylococci have become the most common causes of nosocomial bacterial infections, and this fact, along with increasing problems associated with antimicrobial resistance, spurs the need for finding immunotherapeutic alternatives to prevent and possibly treat these infections. Most virulent, clinical isolates of both coagulase-negative staphylococci (CoNS) and *Staphylococcus aureus* carry the *ica* locus which encodes proteins that synthesize a polymer of β-1-6 linked *N*-acetyl glucosamine residues (PNAG). Animal studies have shown purified PNAG can elicit protective immunity against both CoNS and *S. aureus*, suggesting its potential as a broadly protective vaccine for many clinically important strains of staphylococci.
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1. Introduction

In the past 25 years Gram-positive cocci in general, and staphylococci in particular, have become the primary bacterial organisms isolated from nosocomial infections [1,2]. Associated with this increase in occurrence is the increase in antimicrobial resistance [3,4] which has lead to intense interest in alternative strategies to prevent and control infection. One obvious approach is the development of immunotherapeutics that could be used prophylactically for prevention of infection in high risk patients and possibly therapeutically as an adjunct for standard antibiotic therapy. The challenge of developing such reagents lies principally in identification of antigenic targets for vaccines and definition of immune effectors that mediate resistance to infection.

For extant vaccines that prevent bacterial infections by targeting the killing of the microbial cell, surface polysaccharides have been the most effective. Usually these are referred to as capsular polysaccharides, and immunogenic polysaccharides or protein-polysaccharide conjugates from *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis* have proven highly successful in controlling infections due to these pathogens [5–10]. Many comparable vaccines based on surface polysaccharides are being developed for bacterial pathogens such as group B streptococcus [11], *Klebsiella pneumoniae* [12], *Enterococci* [13,14] and *Pseudomonas aeruginosa* [15,16]. This is predicated upon the strong consensus that when it is feasible to induce immunity to bacterial surface polysaccharides this usually results in the most effective vaccine.

For *Staphylococcus aureus*, two major groups of surface polysaccharides have been identified and targeted for vaccine development. Work by Karakawa and co-workers [17–19] established a capsule typing system for *S. aureus* composed on 11 different serologic types. Two of these, types 1 and 2, appear to be expressed by only individual clones of *S. aureus* and are not found among clinical isolates [20,21]. However, for the remaining nine serotypes, only two of these, types 5 and 8, have actually been shown to be antigens that represent serologically distinct capsules [17,19]. There is no antigenic or definitive serologic characterizations for any of the other capsule types that indicates they are distinct surface polysaccharides. However, the vast majority of isolates of *S. aureus* express either the type 5 or type 8 capsule, making these reasonable targets for vaccine development. Indeed, intense interest has been focused on such development [22–27] and a recent clinical trial of a bivalent type5/type 8 conjugate vaccine given to hemodialysis patients showed a reduction in rates of bacteremia during the early phases of the study, but this reduction was not maintained at the conclusion of the study at 54 weeks [22].

A second surface polysaccharide, found on both *S. aureus* and *S. epidermidis*, is a poly-*N*-acetyl glucosamine (PNAG)

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antigen associated with a number of important biologic and pathologic properties of these organisms [28–34]. The antigen was first described by Tojo et al. [28] as the capsular polysaccharide/adhesin (PS/A) of *S. epidermidis* although a definitive chemical composition and structure was not given. The first report on the chemical properties of this antigen came from Mack et al. [33] who had previously attributed to this antigen the property of mediating intercellular adherence of coagulase-negative staphylococci (CoNS) and named the factor the polysaccharide intercellular adhesin (PIA). Later on McKenney et al. found the same material expressed in *S. aureus* [35] although they mistakenly identified *N*-acetyl succinate as a major component of the vaccine. Recent studies have corrected this misidentification [36] and attributed it to the generation of a degradation product of the PNAG molecule that was produced during acid hydrolysis in order to perform NMR determinations of the structure of PS/A [37]. Another variant of the PNAG polymer was described as the slime-associated antigen (SAA) [38] which was reported to contain about 70% glucosamine. Likely the rest of the material was contaminants. Finally, Rupp and Archer described a hemagglutinin of *S. epidermidis* [39] which was later shown to be PIA [40]. There is now clear consensus that PS/A, PIA and SAA are all chemically PNAG.

1.1. PS/A, PIA and PNAG-relatedness of their chemical and biological properties

PS/A was identified by immunologic means as a capsule of many important clinical isolates of CoNS that had the property of forming a biofilm or producing “slime” in vitro when grown on plastic or glass [28]. Christensen and co-workers [41–44] were instrumental in identifying slime-producing CoNS as major causes of biomedical device infections starting in the early 1980s. Isolation of PS/A identified a major factor in the slime whose properties appeared to promote adherence of bacteria to plastic and formation of a biofilm [28]. Transposon mutants were identified that lost production of PS/A [45], but the exact genes that were interrupted were never identified. The PS/A mutants were found to have reduced virulence in models of endocarditis [46,47] and expression of PS/A antigen was needed to promote resistance of CoNS to innate opsonic factors.

PIA was first described in 1992 by Mack et al. [48] as a factor whose expression was induced by glucose leading to increased intercellular adhesion among CoNS. A genetic locus in CoNS involved in production of a hexosamine polysaccharide involved in intercellular accumulation was then identified in a strain of *S. epidermidis*. PIA was next isolated and purified and then reported to be a small molecular weight (<28 kDa) linear polymer of β-1-6-linked *N*-acetyl glucosamine residues [33] with some O-linked substituents of succinate and phosphate. Heilmann et al. [49] followed this up by identifying the biosynthetic locus for PIA, termed the *ica* locus for intercellular adhesin and initially reported the presence of three open reading frames (ORFs), *ica*, *ica*B, and *ica*C and a divergently transcribed apparent regulator, the *ica*R gene, separated from *ica*A by an approximately 200 base-pair promoter region. A fourth ORF, *ica*D, was then identified [50] whose coding sequence started in the 5′ end of the *ica*A gene and finished in the 3′ beginning of the *Ica*B gene. Expression of the IcaA and icaD proteins in membranes resulted in the synthesis of an oligomer of β-1-6-linked *N*-acetyl glucosamine about 20 residues in length using UDP-*N*-acetyl glucosamine as a starting substrate, and addition of the IcaC protein further increased the oligomer’s size. The role for IcaB remains undefined. Clearly these genes and their protein products are responsible for synthesis of the PIA.

The distinction between PIA and PS/A was founded on the reported inability to show that the *ica* locus was needed for initial adherence of CoNS to plastic tissue culture wells that were manufactured in Europe [51]. However, in the same report, loss of *ica* genes resulted in a loss of adherence of *S. epidermidis* to glass [51]. Thus it was suggested that PS/A mediated the initial adherence of CoNS to plastic and similar surfaces while PIA mediated accumulation of the cells into a biofilm i.e., intercellular accumulation. When McKenney et al. studied the cloned *ica* genes expressed in *S. carnosus* provided by Heilmann et al. [51] they found in fact they could isolate the PS/A material [34] and indicated it was a high molecular weight glucosamine polymer containing N-linked succinate. However, the succinate was subsequently found to have been misidentified [36,37] and, in fact, they had isolated a high molecular weight β-1-6-linked *N*-acetyl glucosamine with evidence of small amounts of O-linked succinate and acetate. Thus, both PS/A and PIA were found to be chemically identical, with some differences reported in the molecular size and larger differences in the biologic functions of these molecules.

As it turned out, the claim that PS/A mediated initial adherence of CoNS to plastic and PIA the accumulation of cells into biofilms was largely predicated on the results of studies with *S. carnosus* carrying the cloned *ica* genes and its interaction with tissue culture wells. When Heilmann performed the biofilm assay on tissue culture plates from the United States (Corning brand) using the identical methods she used in Germany to characterize the *ica* locus, she found that in fact *S. carnosus* carrying the *ica* genes readily formed a biofilm on the plastic plates manufactured in the United States (Fig. 1). Thus, even this distinction between PS/A and PIA was found to be due to some trivial differences in manufacture of tissue culture wells and it is now accepted that PIA and PS/A are the same chemical entity-PNAG.

1.2. Occurrence of ica genes and PNAG-expression in S. aureus

In 1999 McKenney et al. [35] reported that the *ica* genes were present in clinical isolates of *S. aureus* and expression of the PNAG antigen (incorrectly identified as poly-*N*-succinyl glucosamine or PNSG) was mostly

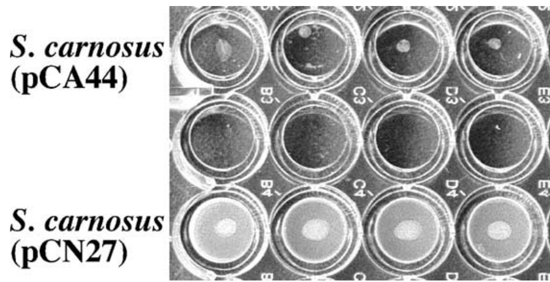

Fig. 1. Biofilm formation in tissue culture wells (Corning brand) by *S. carnosus* carrying a plasmid with the *ica* genes from *S. epidermidis* (pCN27) and expressing PNAG or carrying the plasmid without additional DNA (pCA44). In contrast to the initial report that *S. carnosus* (pCN27) did not make a biofilm on plastic [49] this experiment showed that with the Corning brand of tissue culture plate a biofilm is formed. The inability of *S. carnosus* (pCN27) to form a biofilm on one brand of tissue culture plate was the basis for distinguishing PIA and PS/A, which are now clearly known to be the same molecule.

associated with in vivo growing organisms. However, when grown in vitro in rich medium (brain heart infusion broth) supplemented with glucose, there was increased expression of the PNAG antigen (Fig. 2). They also showed expression of PNAG by *S. aureus* in lung sections from two cystic fibrosis patients and in six of nine sputum samples also from cystic fibrosis patients [35]. Strains of *S. aureus* isolated from infected mice had increased PNAG expression in vitro, but after five passages the expression returned to a low state [35]. Cramton et al. rapidly followed this up with a similar

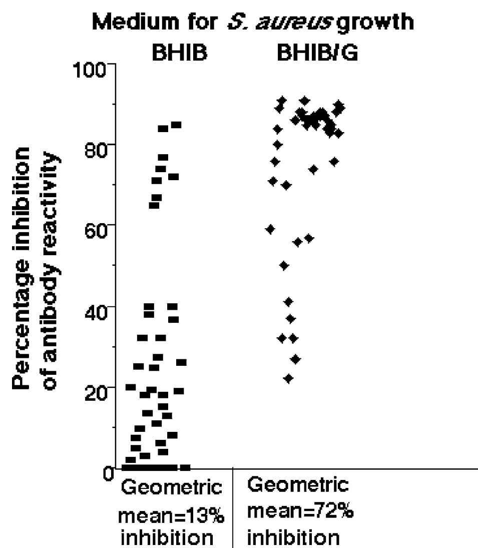

Fig. 2. Induction of expression of PNAG in clinical isolates of *S. aureus* following growth in glucose-supplemented media. Strains were grown in either brain-heart infusion broth (BHIB) or BHIB supplemented with 0.25% glucose (BHIB/G) overnight, cells recovered by centrifugation and used to adsorb out a standard dilution of rabbit antibody to purified PNAG [35]. The antiserum was added to an ELISA plate coated with purified PNAG and the percentage inhibition of antibody binding measured. The geometric mean percentage inhibition of antibody binding, indicative of PNAG-expression, was significantly lower ($P < 0.01$, *t*-test) in strains grown in BHIB compared to those grown in BHIB/G.

report that the *ica* locus was present in *S. aureus* [52] and was needed for biofilm formation by this organism. This report made no distinction between initial adherence and accumulation of cells into biofilms, as both properties in *S. aureus* were affected by deleting most of the *ica* locus. Several subsequent reports confirmed that the *ica* genes were found in most clinical isolates of *S. aureus* [53,54] and those reports that did not find *ica* genes in the majority of isolates [54] were criticized for using primers designed for the *S. epidermidis ica* genes for investigating *S. aureus* [55]. There is about 70–80% identity at the nucleotide level of the *ica* genes in these two species [35,52], so primers based more on *S. aureus* sequences would be optimal for finding these genes in *S. aureus*. Among bovine isolates of *S. aureus* causing mastitis, 100% of 35 strains were found to carry the *ica* genes [56]. Peacock et al. [57] identified seven *S. aureus* genes encoding putative virulence factors out of 33 studied that were strongly associated with invasive strains when compared with strains of *S. aureus* carried by healthy blood donors and the *ica* genes were one of these seven. Thus, the presence of *ica* and the expression of PNAG is strongly associated with virulent strains of *S. aureus* and *S. epidermidis* [31,58,59].

1.3. Role of the PNAG surface polysaccharide in virulence of Staphylococcal infections

Accepting that PS/A, PIA and SAA are all basically PNAG polymers synthesized by proteins encoded by the *ica* locus, there is a fair amount of data that this polymer plays an important role in the virulence of infections due to CoNS. However, outside of epidemiologic associations of the occurrence of the *ica* locus in invasive isolates of *S. aureus* [57], there is surprisingly little information available about the role of PNAG in virulence of this species. Data reported in abstract form [75] indicate a reduced level of virulence of *S. aureus* strains deleted for the *ica* locus when tested in a model of endocarditis in rats (Fig. 3). In this model it was found the infectious dose for 50% (ID_{50}) of the animals infected with the wild type strain was <43 cfu, as all five animals infected with this dose had evidence of endocarditis, while for the *ica*-deleted strain the ID_{50} was 6.9×10^6 cfu ($P < 0.001$, logit analysis). Ten of 24 animals infected with the wild-type strain at doses $\leq 10^{6.3}$ died 7–9 days after infection while none of 16 infected with the mutant strain died ($P < 0.001$, Fisher's exact test). Thus, in endocarditis it appears from this one study that PNAG is a virulence factor for *S. aureus*. In contrast, Francois et al. [60] reported no difference in virulence between wild-type and *ica* deletent *S. aureus* strains in a model of foreign body infection using tissue cages implanted into guinea pigs. However, in this model the cages are first implanted in the animals and left for 3 weeks before infection, allowing the cages to become coated with host proteins. Given the ability of *S. aureus* to bind to numerous host proteins including fibrinogen, fibronectin, collagen and others [61–63] it is not surprising that when confronted with a foreign body coated

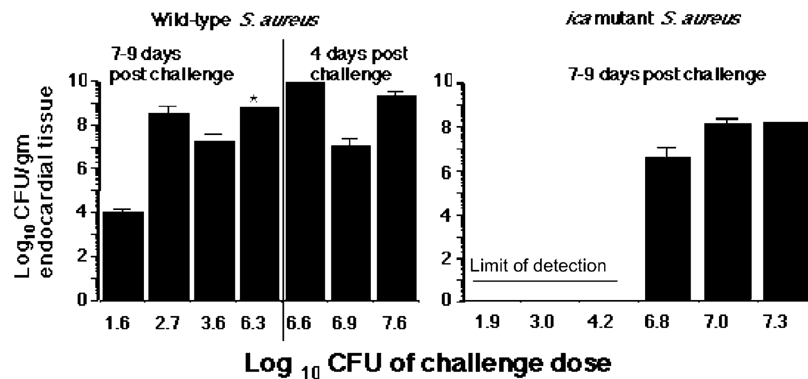

Fig. 3. Virulence of a wild-type and isogenic mutant of *S. aureus* strain 10833 deleted for the *ica* locus in a rat model of endocarditis [23]. Rats with intraaortic catheters were infected with the dose of the wild type or mutant strain indicated on the *X*-axis and sacrificed at the time shown above the data bars, endocardial vegetations identified, excised, weighed, homogenized and serial dilutions plated for bacterial enumeration. The lower limit of detection (10 cfu/vegetation) is indicated and rats challenged with the indicated doses had no detectable vegetations or bacteria in their hearts. Bars represent means and error bars the S.E.M. Rats challenged with the higher doses of the wild-type strain had to be sacrificed early as they would not survive a longer period, further illustrating the enhanced virulence of the wild type strain compared to the *ica*-mutant in this model of infection. By comparative analysis of the overall cfu/gm of vegetation achieved, regardless of the day of sacrifice, it took approximately 4 logs more of the *ica*-deleted strain to reach comparable vegetation levels as did the wild type parental strain.

with host proteins the surface PNAG is not required for adherence and biofilm formation and thus a role in virulence may not be manifest in this setting.

Early studies on biofilm-producing phenotypic variants of *S. epidermidis* [64,65] indicated that the variants unable to make a strong biofilm were less virulent in a mouse model of foreign body infection. In contrast, Patrick et al. [66] suggested in vitro slime production was not necessarily associated with pathogenesis of CoNS, particularly in the absence of a foreign body. A later study in mice showed wide heterogeneity in the ability of strains of CoNS with different biofilm phenotypes to produce infections [67] but concluded there was some association between biofilm elaboration and virulence. Deighton et al. [68] compared the virulence of five biofilm-positive and five biofilm-negative strains in a mouse abscess model without a foreign body implanted and found the biofilm-positive strains caused more abscesses that persisted longer with higher bacterial counts compared with the five biofilm-negative strains. However, these studies were conducted without knowledge as to the biochemical or genetic basis for biofilm production and classifying strains as biofilm positive or negative was based on in vitro measurements, which are known to vary widely based on conditions used to asses biofilm formation.

Subsequent studies with genetically manipulated strains of *S. epidermidis* gave more conclusive data that the biofilm-positive phenotype was associated with virulence. Transposon mutants of *S. aureus* strain M187 that lead to a biofilm-negative phenotype [45] were found to be avirulent in a rabbit model of endocarditis [46] following high dose-inoculation, and similarly were poorly virulent in a model of endocarditis following hematogenous spread from a contaminated intravascular catheter [47]. These studies focused on the role of the PNAG-polymer as an antiphagocytic bacterial capsule, that in addition to promoting adherence of Staphylococci to biomaterials also prevented opsonic killing due to endogenous complement and phagocytic activity. However, Perdreau-Remington et al. [69] did not find any difference in virulence in a rabbit model of endocarditis when comparing the strong biofilm-producing *S. epidermidis* strain RP62A with a chemical mutant deficient in production of biofilm. In a rat model of intravenous catheter associated infection [70], Rupp et al. [71] showed that there was less infection with a mutant of *S. epidermidis* strain 1457 unable to make the PNAG polymer compared with the parental strain. Another study showed the same effect with an *ica* mutant in strain O-47 [72]. In a related model of foreign body infections in mice, the same strain of *S. epidermidis* deficient in production of biofilms caused fewer abscesses and adhered to the implanted foreign body less well than did the parental strain. Overall, the general consensus from these studies is that elaboration of the PNAG polymer by CoNS, particularly *S. epidermidis*, is not only epidemiologically associated with pathogenic strains [73] but plays an important role in virulence as determined by animal studies.

1.4. Role of the PNAG surface polysaccharide in vaccination

As *ica* genes and PNAG-expression are found commonly among clinical isolates of both CoNS and *S. aureus*, it is obviously an attractive vaccine candidate with the potential to elicit immunity to both of these common causes of nosocomial infection. As this polymer was first identified as PS/A in CoNS, the first studies on the vaccine potential of the PNAG polymer were performed with the PS/A material, although the level of purity of the vaccine could not be ascertained as its chemical nature was not known at the time. Nonetheless, from subsequent studies it is highly likely that the major

component of the vaccine was PNAG. This immunogen was shown to reduce the number of days that rabbits had positive blood cultures, in comparison to non-immune controls, in a model of catheter-related bacteremia [29]. Passive therapy using polyclonal and monoclonal antibody to the polymer also conferred protection. In a rabbit model of endocarditis, immunization with the PS/A/PNAG polymer also markedly reduced the rate of occurrence of positive blood cultures and protected against the development of infected vegetations [30]. When it was discovered that the *ica* locus was present in most isolates of *S. aureus* and PNAG was expressed, it was also found that active or passive immunization protected mice against infection with eight different clinical isolates in a kidney infection model [35]. Additionally, rabbit antisera raised to purified PNAG has shown passive protective efficacy against infection in a rat model of endocarditis using a wild-type strain of *S. aureus* but not an isogenic strain deleted for the *ica* locus (Fig. 4). In this experiment, rats with intra-aortic catheters were challenged with *S. aureus* strain 10833 with either an intact or deleted *ica* locus; the challenge dose for the wild-type strain was 2×10^4 cfu per rat whereas for the less virulent mutant strain the challenge dose had to be 9×10^6 cfu per rat in order to achieve comparable levels of infected vegetations with these two strains. Four days after infection animals were sacrificed and vegetations identified, excised, weighed and homogenized for bacterial levels. Immune serum to PNAG significantly ($P = 0.0014$, *t*-test) reduced the bacterial levels in vegetations in rats infected with the wild type strain but had no effect in animals infected with the *ica* deletent (Fig. 4). All seven of the animals infected with the wild-type strain and treated passively with normal rabbit serum had infected vegetations compared with only three of eight animals treated with immune serum ($P = 0.02$, Fisher's exact test). This experiment provided additional data indicating the potential of antibody to PNAG to protect against *S. aureus* infection and also showed the specificity of the protection in regard to the inability to protect against infection with the strain lacking an intact *ica* locus.

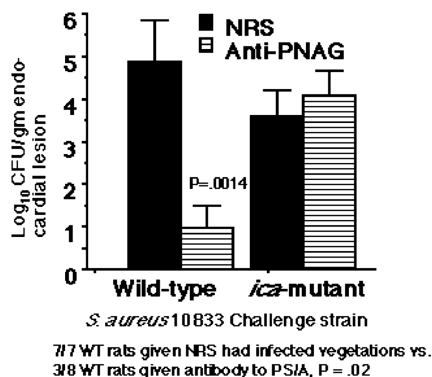

Fig. 4. Passive protection mediated by rabbit antibody to purified PNAG in a rat model of endocarditis. Animals with intraaortic catheters were treated with 0.5 ml of either normal (NRS) or immune serum to PNAG and then infected with either 2×10^4 cfu/rat of the wild-type, parental strain or 9×10^6 cfu/rat for the less virulent *ica*-mutant strain. This higher challenge dose for the mutant strain was needed in order to achieve comparable levels of infection in the aortic valve vegetations. Four days later animals were sacrificed and levels of bacteria in the vegetations determined. Bars represent means and error bars the S.E.M.

Although to date there are the only three published studies in the peer-reviewed literature on the vaccine potential of PNAG, there is continued on-going work on the immunochemical properties of the antigen to enhance immunogenicity and protective efficacy. A recent abstract [76] indicated that conjugating PNAG to diphtheria toxoid enhanced its immunogenicity in mice and rabbits compared with antibody levels obtained using unconjugated PNAG [36]. The antisera had opsonic killing activity against a variety of *S. aureus* strains and one *S. epidermidis* strain. Another abstract [77] showed that antibodies to PNAG were produced by cystic fibrosis patients with staphylococcal colonization or infection, indicating that the antigen was expressed in vivo at a sufficient level to induce antibody. Overall, continued work on a PNAG vaccine is progressing, with both direct animal studies and correlative studies on responses of infected humans on-going, with the ultimate goal of a clinical assessment of active and passive immunotherapies directed at this antigen.

2. Conclusion

It is now clear that the various forms of staphylococcal surface polysaccharides identified as PS/A, PIA and SAA are the same chemical entity-PNAG. The structure was first identified by W. Fischer as reported by Mack et al. [33] although the material isolated in this case was of a small molecular weight. Papers describing an N-linked succinate component [34,35] were incorrect in this identification [36,37]. The biosynthetic proteins for PNAG are encoded by the *ica* locus first identified by Heilmann et al. [49] in *S. epidermidis* and subsequently by McKenney et al. in *S. aureus* [35] followed shortly thereafter by Cramton et al. [52]. Studies in *S. epidermidis* and other CoNS show a clear association of PNAG production and virulence based on both epidemiologic studies of clinical isolates and animal studies of phenotypic variants and genetic mutants. Immunization with PNAG protected against infection in rabbits due to catheter-associated bacteremia [29] and endocarditis [30]. In *S. aureus*, PNAG production is found in virtually all clinical isolates and immunization has been reported to protect mice against infection caused by up to eight different clinical isolates [35]. PNAG purified from an over-producing mutant of *S. aureus* strain MN8 [74] is immunogenic in laboratory animals [36] and work reported in abstract form indicates conjugation of PNAG to carrier proteins enhances immunogenicity. Further studies in different animal models and identification of the optimal form of PNAG for testing in animal, and eventually human, immunogenicity studies is clearly warranted and if the proper types of immune effectors mediating resistance can be identified then there is a

potential for PNAG to mediate protective immunity against the majority of virulent strains of CoNS and *S. aureus*.

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