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Capsular polysaccharides are an important immune evasion mechanism for *Staphylococcus aureus*

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Abbreviations: OPA, opsonophagocytic antibody assay; OP, opsonophagocytic; BRC, baby rabbit complement; CP, capsular polysaccharide; CP5, capsular polysaccharide type 5; CP8, capsular polysaccharide type 8; MRSA, methicillin resistant *S. aureus*; MSSA, methicillin sensitive *S. aureus*; CFU, colony forming units; MFI, mean fluorescence intensity; SpA, staphylococcal protein A

Staphylococcus aureus can cause severe life threatening invasive diseases. The principal immune effector mechanism by which humans are protected from Gram positive bacteria such as *S. aureus* is antigen specific antibody- and complement-dependent opsonophagocytosis. This process can be measured in vitro using the opsonophagocytic antibody assay (OPA), which is a complex assay composed of live *S. aureus* bacteria, a complement source, phagocytic effector cells such as differentiated HL-60 cells, and test serum. In this report, we investigated the impact on the OPA of *S. aureus* surface antigens capsular polysaccharides (CP) and protein A (SpA). We demonstrated that higher CP expression renders bacteria more resistant to non-specific opsonophagocytic killing than increased SpA expression, suggesting that the expression of capsular polysaccharides may be the more important immune evasion strategy for *S. aureus*. Bacteria that were not fully encapsulated were highly susceptible to non-specific killing in the assay in the absence of immune serum. This non-specific killing was prevented by growing the bacteria under conditions that increased capsular polysaccharide levels on the surface of the bacteria. In contrast, the level of SpA expression had no detectable effect on non-specific killing in OPA. Using anti-CP antibodies we demonstrated type-specific killing in OPA of both MRSA and MSSA clinical isolates. SpA expression on the cell surface did not interfere with OPA activity, providing evidence that despite the role of SpA in sequestering antibodies by their Fc region, killing is easily accomplished in the presence of high titered anti-capsular polysaccharide antibodies. This highlights the role of CP as an important immune evasion mechanism and supports the inclusion of capsular polysaccharide antigens in the formulation of multi-component prophylactic vaccines against *S. aureus*.

Introduction

Staphylococcus aureus are Gram-positive commensal bacteria that asymptomatically colonize ~30% of humans.[1] A breach in the skin or other mucosal barriers allows *S. aureus* to invade the host and cause diseases ranging from mild skin infection to more devastating conditions such as bacteremia, osteomyelitis and endocarditis.[2-4] Antibiotic resistant *S. aureus* such as methicillin resistant *S. aureus* (MRSA) strains in both hospital and community settings have reduced treatment options and underscore the need for a prophylactic vaccine to prevent such infections and associated diseases.

Similar to other bacterial pathogens, *S. aureus* expresses capsular polysaccharide (CP) as a virulence factor to avoid phagocytic killing.[5] There are two major capsular polysaccharide types, CP5 and CP8 and all clinical *S. aureus* strains have the biosynthetic pathways for making either CP5 or CP8.[6] Over the past decade, an extensive collection of published preclinical data has emerged that demonstrates the efficacy of CP conjugate vaccines in different animal models.[7-9] The mechanism of protection conferred by capsule-specific functional antibodies is mainly due to their role in mediating opsonophagocytosis.

Protein A (SpA) is another virulence factor expressed by *S. aureus* that is thought to inhibit OP killing by neutrophils.[10] SpA can prevent phagocytosis by binding the complement binding portion (Fcγ) of functional antibodies thus hindering the antibody binding to bacterial cell surface components and subsequent complement dependent uptake of bacteria by phagocytic cells.

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Table 1. Capsule expression reduces non-specific kill of *S. aureus* strains in OPA

Strain	Growth time (hr)[a]	Control IgG (MFI)	CP5 (MFI)[b]	CP8 (MFI)[c]	Protein A (MFI)	%kill[d]
PFESA0158 (CP5)	4	163	283		972	-61
	6	372	472		953	-54
	22	649	8,858		966	-30
PFESA0186 (CP8)	4	85		574	32,389	-30
	6	101		621	34,015	-28
	22	160		28,378	28,999	-7

[a]PFESA0158 and PFESA0186 were grown in TSB to different growth phase and their CP expression levels were determined using flow cytometry. [b]Strain PFESA0158 was stained with CP5 mAb and compared with isotype matched mIgG as control. [c]Strain PFESA0186 was stained with CP8 mAb and compared with isotype matched mIgG as control. [d]Negative number denotes non-specific kill. Data are average of four independent experiments.

Opsonophagocytic antibody assays (OPAs) have been used as a correlate of protection for the evaluation of *Streptococcus pneumoniae* conjugate vaccine responses[11] and can also be used to assess function of anti-staphylococcal antibodies.[12] These assays are designed to quantify functional antibodies in human serum samples that enable phagocytic effector cells (such as polymorphonuclear leukocytes or differentiated HL-60 cells) to recognize and destroy bacteria in the presence of a complement source. An in vitro opsonic reaction is initiated when bacterial cells, human serum (heat inactivated to abrogate endogenous complement activity) containing functional antibodies to the bacteria, and an exogenous complement source are mixed together. Complement-receptor and Fc- receptor bearing phagocytic effector cells can then engulf and kill the opsonized (antibody and complement coated) bacteria. An OPA titer is expressed as the reciprocal of the highest serum dilution that results in the effector cell and complement dependent killing of 50% of the target bacteria in the assay.

In this study, we investigated whether in vitro OP killing of *S. aureus* by functional antibodies to CP5 or CP8 could be influenced by the level of surface expression of the CPs and whether the expression level of SpA could potentially interfere with this activity. We also sought to carefully examine the interplay of surface antigen expression levels and complement concentrations in OP killing of *S. aureus* including factors leading to non-specific (background) killing. Non-specific killing is the killing of the target bacteria in the absence of test serum that can result from the presence of antibodies and/or other factors in the complement source. Our results show that capsular polysaccharide but not SpA expression prevents nonspecific killing of *S. aureus* and that both MSSA and MRSA could be killed by anti-CP antibodies irrespective of SpA antigen levels.

Results

Both CP5 and CP8 protect *S. aureus* from non-specific killing in OPA. Expression of capsular polysaccharide is a common mechanism by which *S. aureus* and other pathogenic bacteria evade opsonophagocytosis. To assess the role of capsule expression in preventing non-specific killing, two *S. aureus* clinical isolates (PFESA0158 and PFESA0186 expressing CP5 or CP8, respectively) were grown under conditions that resulted in varied capsule expression levels. The bacteria were then tested in the OPA, using 2% complement in the absence of immune serum. Both strains were tested after 4–6 h growth when low or negligible levels of capsule expression were detected or after overnight culture that resulted in high levels of capsule expression. PFESA0158 samples with no capsule expression showed non-specific killing ranging between 54 and 61% (**Table 1**). Non-specific killing for PFESA0186 with low levels of capsule expression ranged between 28 and 30%. After overnight culturing, both CP5 and CP8 strains exhibited high capsule expression and demonstrated reduced non-specific killing of 30 and 7%, respectively.

Next we examined the effect of varying complement concentration on non-specific killing of *S. aureus* strains PFESA0158 and PFESA0186, grown to express high levels of capsule to render bacteria more resistant to in vitro OP killing. Complement concentrations ≥ 5% resulted in substantial non-specific killing (≥ 45%) for both strains (**Fig. 1A and B**). These results demonstrate that high complement concentrations can cause extensive reduction of bacteria in OPA even in the presence of high capsular polysaccharide expression and support the importance of optimizing this critical parameter.

We also assessed the correlation between high capsule expression and increased resistance to in vitro non-specific killing for six MRSA strain using 1% complement concentration (**Table 2**). As shown with the two MSSA strains PFESA0158 and PFESA0186, highly encapsulated MRSA strains were also resistant to non-specific killing in OPA. In contrast to the non-encapsulated bacteria, bacterial growth as opposed to non-specific killing was observed for the highly encapsulated strains during the duration of the assay. For the CP5 strains, CFUs increased by 48% to 61% during the 60 min incubation in the OPA while CP8 strain CFUs increased by ≤ 28% (**Table 2**). These results clearly demonstrate that high capsule expression not only renders the bacteria resistant to opsonophagocytic killing as measured in OPA using 1% complement concentration but furthermore allows the bacteria (best demonstrated by the CP5 expressing strains) to grow under these conditions.

Anti-capsular polysaccharide antibodies kill encapsulated *S. aureus* cells in OPA. After demonstration that capsular polysaccharide expression is important for *S. aureus* to evade opsonophagocytic killing, we explored whether immunization with CP5 or CP8 polysaccharide conjugate vaccines would elicit functional anti-capsular antibodies that can kill *S. aureus* in OPA. A panel of sera from rhesus macaques immunized with CP5 or CP8

conjugate vaccines were tested using strains PFESA0046 (CP5), PFESA0048 (CP5) and PFESA0186 (CP8). Four non-immune sera were included as negative controls. Titratable CP5 and CP8 OP activity was observed for the immune serum samples, as evident from the kill curves in **Figures 2 and 3**, respectively. Geometric mean titers and 95% confidence intervals were calculated for each sample as shown in **Tables 3 and 4**. In both CP5 and CP8 OPA, the four non-immune samples lacked any detectable OP activity and were arbitrarily assigned a titer value of 50 (half the value of the lowest serum dilution tested in the assay). Comparable CP5 OP activity was observed for most samples against both MRSA strains. Overall, robust and reproducible OP titers were observed for both serotypes.

Specificity of the anti-CP5 antibodies for the CP5 expressing MRSA strains was demonstrated by complete inhibition of the OP activity in the presence of homologous (CP5) but not heterologous (CP8) competitor polysaccharide (**Fig. 4**). OPA kill curves for the samples without competitor or heterologous CP8 competitor were indistinguishable. Similarly, specificity of the anti-CP8 antibodies was also confirmed by complete inhibition of the OP activity in the presence of homologous competitor (data not shown). These results demonstrate that anti-capsular antibodies elicited after immunization with capsular polysaccharide conjugate vaccines are functional and kill MRSA strains in the presence of complement and differentiated effector cells.

SpA does not inhibit OPA killing. SpA has been extensively studied as an immune evasion mechanism inhibiting phagocytosis. To examine whether SpA expression has an impact on bacterial survival in an in vitro OPA, we first tested whether clinical isolates do express SpA under conditions that are conducive to capsular polysaccharide expression. As shown in **Table 2**, SpA expression was observed for 4 of 8 of the *S. aureus* clinical isolates tested. Strains PFESA0046 and PFESA0048 were effectively killed in OPA by CP5 conjugate vaccine elicited immune sera irrespective of SpA expression (**Table 3**). An effect of growth media on SpA expression was observed for overnight grown PFESA0186 cultures with antigen detected in TSB but not modified Frantz media as shown in **Tables 1 and 2**. In addition, our results show that SpA expression did not correlate with non-specific killing as exemplified by strain PFESA0186 (**Table 1**). High levels of SpA expression was observed at all time points whereas high capsule expression was only observed at 22-h time point which closely correlated with lowest level of non-specific killing of bacteria. Together these data show that neither the presence of SpA nor the expression of capsular polysaccharides on the surface of staphylococci interfere with the ability of functional anti-CP antibodies to mediate opsonophagocytic killing of *S. aureus* (**Fig. 2**).

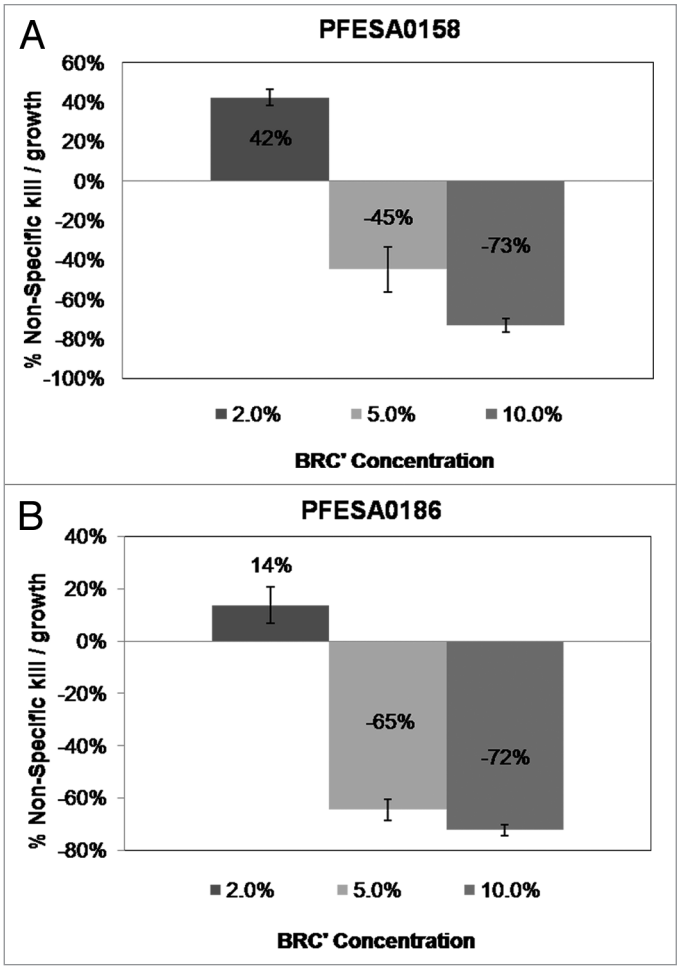

Figure 1. Enhanced non-specific killing with increasing complement concentration in OPA. (**A**) PFESA0158 and (**B**) PFESA0186 strains were incubated with either 2%, 5% or 10% complement plus HL60 cells at 37°C for 60 min. Negative and positive percentage in the bar graphs denotes kill and growth, respectively. The surviving bacteria were counted using a CTL immunospot® reader. Percent kill/growth was calculated by comparing the bacterial counts at T0 (input) and at 60 min post-phagocytosis incubation step. Each bar is average of four independent experiments ± standard deviation.

Discussion

Opsonophagocytic killing by PMNs is the primary mechanism by which clearance of *S. aureus* is performed by the host immune system.[14-16] Patients with neutropenia or with congenital or acquired neutrophil functional impairment are highly susceptible to *S. aureus* infections.[17-21] A critical role for functional antibodies and the complement system has also been established in reducing *S. aureus* levels both in in vitro and in vivo settings.[22-26] Given the importance of the opsonophagocytosis mechanism for clearance of *S. aureus* in vivo it is important to develop a robust and specific in vitro OPA to determine whether vaccine induced antibodies are functional and can kill *S. aureus*. To this end, we showed the importance of complement concentration as a critical parameter in OPA to ascertain that non-specific killing is reduced so that functional antibody dependent killing can be reliably measured in serum samples. Substantial non-specific kill of bacteria was observed at complement concentrations ≥ 5% that can contribute to variability in the OPA data (**Fig. 1**). Additionally, high non-specific kill makes it difficult to dissect whether the OPA response is mediated by test serum antibody or other, non-specific factors.

Table 2. MRSA and MSSA strains expressing high levels of capsule are less susceptible to non-specific killing in OPA

Strain	Capsular type	Methicillin susceptibility	Control IgG (MFI)	CP5 (MFI)[b]	CP8 (MFI)[c]	Protein A (MFI)	% growth[d]
PFESA0158	5	MSSA	54	20,000		64	48
PFESA0046	5	MRSA	37	9,887		633	61
PFESA0048	5	MRSA	42	16,400		1,205	55
PFESA0054	5	MRSA	161	23,500		61	61
PFESA0186	8	MSSA	36		33,300	38	24
PFESA0012	8	MRSA	70		21,300	41	6
PFESA0110	8	MRSA	62		18,700	5,017	-4
PFESA0120	8	MRSA	101		20,600	3,062	28

[a]Strains were grown overnight in modified Frantz media and their CP expression levels were determined using flow cytometry. [b]CP5 strains were stained with CP5 mAb and compared with isotype matched mIgG control. [c]CP8 strains were stained with CP8 mAb and compared with isotype matched mIgG control. [d]Positive number denotes growth.

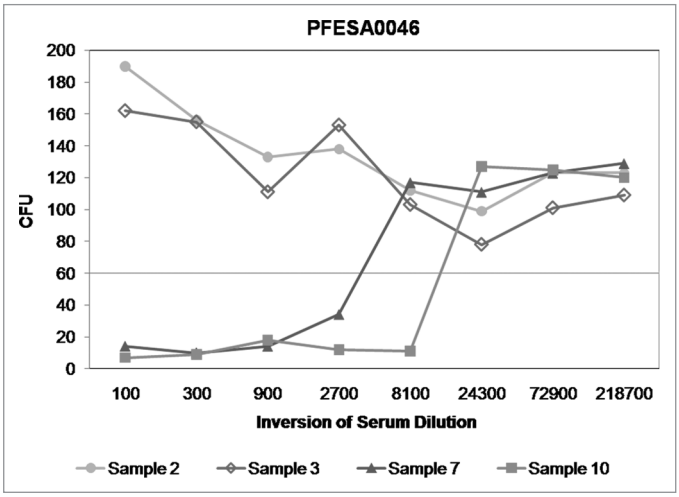

Figure 2. CP5 conjugate vaccine induced immune sera that effectively kill MRSA strain PFESA0046. Sera samples (7 and 8) from rhesus macaques vaccinated with the CP5-CRM_{197} conjugate was tested for OP activity against PFESA0046 strain. Non-vaccinated serum samples (2 and 3) were used as negative controls for the assay. The titer is defined as the reciprocal of the highest serum dilution that kills 50% of the test bacteria. The CFU associated with 50% bacterial killing is indicated by the horizontal line.

There are at least two mechanisms by which *S. aureus* can be opsonized and non-specifically killed in the OPA in a complement concentration-dependent manner; (1) by the presence of pre-existing antibodies in serum used as complement source and (2) by opsonization mediated by C3b deposition. In a previous report by Xu et al. substantial non-specific killing for both CP5 and CP8 strains were observed in the presence of 10% normal rabbit serum.[27] Non-specific killing was marginally reduced when the normal rabbit serum concentration was decreased to 1%. Heat inactivated normal rabbit serum was shown to be poorly opsonic; thereby the authors concluded that C3b deposition was the major opsonization factor involved in the killing process as there was little pre-existing antibodies present in the complement source. Our results corroborate their findings that complement concentrations greater than 5% in OPA cause substantial non-specific killing of the bacteria. Since the opsonic activity of heat inactivated rabbit complement was not evaluated in our experiments our results cannot distinguish between the two non-specific killing mechanisms. The effect of optimal complement concentration in an antibody-mediated (to *S. aureus* surface targets) in vitro killing assay has not been completely appreciated. In a recent report, antibodies to *S. aureus* protein antigen FhuD2 were shown to be opsonic in an OPA using a 10% complement concentration.[28] There was significant non-specific complement-mediated kill observed, making it difficult to assess the contribution of the antigen specific antibody mediated killing. It is imperative that complement concentration in an in vitro assay is optimized to accurately measure the opsonic activity of antibodies generated to vaccine antigens.

Thakker et al., using a laboratory strain of *S. aureus* (Reynolds), showed that the expression of CP5 allows the bacteria to evade opsonophagocytosis compared with strains for which the capsule pathway had been deleted.[5] Our results also demonstrate the protective effect of both CP5 and CP8 expression to resist non-specific killing (i.e., in the absence of immune serum) in the OPA. Both MRSA and MSSA strains grown to express high levels of capsule were resistant to non-specific killing (**Table 2**). Addition of immune serum in the assay clearly demonstrated the opsonophagocytic activity of both anti-CP5 and CP8 antibodies against *S. aureus* clinical isolates (**Figs. 2 and 3**). These results provide support that capsular polysaccharide conjugates are important components to include in a multi-component vaccine to prevent *S. aureus* infections.[12,29]

We also investigated the effect of SpA expression on OPA. Previous reports have questioned whether humoral immunity can be effective at preventing *S. aureus* disease, due to the role of SpA in binding antibodies such that phagocytosis by neutrophils is inhibited.[30,31] Our results demonstrate that antibodies generated against CP are effective in killing strains expressing the SpA antigen thereby supporting that vaccine induced functional antibodies to capsular polysaccharides can be effective in preventing *S. aureus* infection. The effectiveness of anti-CP antibodies in killing SpA expressing bacteria could be due to the conjugate vaccine induced generation of antibodies with high titers and avidity. In contrast, normal human sera containing low titer and

less avid antibodies to *S. aureus* antigens may be more prone to interference by SpA expression. Interference in the opsonic activity of low avidity pre-existing antibodies to CP has been recently reported by Skurnik et al.[32] In this report, the authors provide evidence that presence of natural non-opsonic antibodies to poly-N-acetyl glucosamine (PNAG) in normal human serum interfere with the opsonic activity of antibodies to *S. aureus* CPs detected in subjects who had not been vaccinated with a capsular polysaccharide vaccine.

It is likely that natural exposure to *S. aureus* leads to the generation of antibodies with low avidity that are prone to interference effects by SpA or other factors. The differences between immune responses elicited after *S. aureus* exposure (infection or carriage) compared with the immune responses elicited after immunization with a subunit vaccine have been exemplified by Hawkins et al.[33] In this study non-human primates (NHPs) immunized with *S. aureus* cells mounted a non-functional immune response to Clumping factor A (ClfA) antigen whereas NHPs vaccinated with recombinant ClfA demonstrated a potent functional immune response.

Our results show the importance of optimizing critical OPA parameters such as bacterial capsule expression and complement concentration to avoid non-specific killing and to reliably measure opsonophagocytic killing of bacteria by functional antibodies elicited after immunization with candidate vaccine antigens. By using optimal complement concentration, specific and robust opsonophagocytic killing activity mediated by anti-CP antibodies were shown against MRSA and MSSA clinical strains, and further confirm the role of capsular polysaccharides as *S. aureus* virulence factors in their ability to evade phagocytosis in the absence of such capsule specific antibodies.

Methods

Bacteria. *S. aureus* strains PFESA0158, PFESA0046, PFESA0048, PFESA0054, PFESA0186, PFESA0012, PFESA0110 and PFESA0120 were grown overnight in 100 ml of either tryptic soy broth or modified Frantz media[13] in a shaker incubator at 37°C, and 200 rpm. Overnight grown bacteria were diluted to a starting optical density (OD_{600}) of ~0.3 in 500 ml of fresh medium and incubated at 37°C and 200 rpm. Bacterial samples were harvested at 4-, 6- and 22-h time points. Harvested bacteria were centrifuged and re-suspended to an OD_{600} of ~1 in 30 ml phosphate buffered saline (Mediatech, Cat. #21–031-CV) containing 14% glycerol.

Non-immune and immune sera. Animal sera were generated according to Pfizer's laboratory animal care and use policy, which reflects an absolute commitment that animals used are treated humanely and that such research is conducted only after appropriate ethical consideration and review. Immune sera were generated by injecting rhesus macaques intramuscularly with a single dose of 10, 20 or 100 μg of either CP5 (CP5- CRM_{197}) or CP8 (CP8- CRM_{197}) polysaccharide conjugate vaccine where the polysaccharide was chemically conjugated to the detoxified diptheria toxin CRM_{197}. Each dose of the conjugate was formulated with 250 μg AlPO4. Sera were collected at week 0 (non-immune) or weeks 2, 4 and 6, respectively.

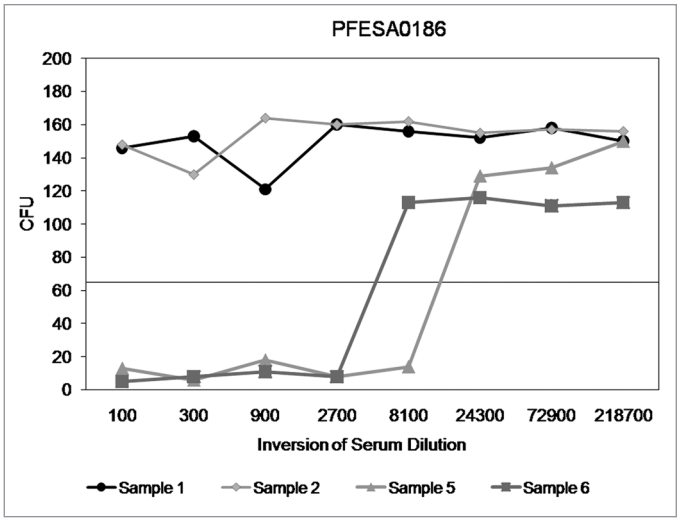

Figure 3. CP8 conjugate vaccine induced immune sera that effectively kill MSSA strain PFESA0186. Sera samples (5 and 6) from rhesus macaques vaccinated with the CP8-CRM_{197} conjugate was tested for OP activity against PFESA0186 strain. Non-vaccinated serum samples (1 and 2) were used as negative controls for the assay. The titer is defined as the reciprocal of the highest serum dilution that kills 50% of the test bacteria. The CFU associated with 50% bacterial killing is indicated by the horizontal line.

Flow cytometry. Monoclonal antibodies that were specific to each of the test antigens CP5 and CP8 polysaccharides (Scully et al. in preparation) and SpA (Sigma, Cat. #P2921) were used as primary antibodies for the detection of surface expression by flow cytometry. Bacterial cells were heat-killed for 1 h at 60°C and then blocked with 10% porcine serum/PBS. Monoclonal antibodies (20 μg/ml) were added and incubated for 30 min on ice. Mouse IgG (Jackson Immunoresearch, Cat. #015-000-003) at a concentration of 20 μg/ml was included as a negative control. Bacteria were subsequently incubated with 10 μg/ml biotinylated anti-mouse IgG (Jackson Immunoresearch, Cat. #115-065-164), followed by 5 μg/ml streptavidin-PE (BD Biosciences, Cat. #554061). Bacteria were washed after each incubation with 10% porcine serum/PBS and after two final washes, cell pellets were resuspended in 1% paraformaldehyde. For each sample, 20,000 events were acquired on a BD LSR II flow cytometer and analyzed using FlowJo v10 software (Treestar, Ashland, OR). The mean fluorescence intensity (MFI) of the PE channel was determined for each sample after gating on bacterial cells in the logarithmic FSC vs. SSC dot plot. A sample was considered positive if the MFI value was at least three times the control mouse IgG and greater than 100.

Opsonophagocytic assay. OPAs were performed as previously described.[12] Baby rabbit complement (BRC) was used as a source of complement in the assay (Pel-Freez, Cat. #: 31061–3). For complement titration experiments, the complement was either undiluted or diluted in assay buffer to 50% or 20% concentration and 10 μl was added to the bacterial mix for a final complement concentration of 10%, 5% and 2% respectively.

Non-specific killing or bacterial growth was calculated by determining the ratio of the number of bacterial colony forming

Table 3. OPA titers against MRSA strains expressing CP5 and SpA antigen

	PFESA0046[d]			PFESA0048[e]		
Sample #[a]	GMT[b]	95% CI Lower	95% CI Upper	GMT	95% CI Lower	95% CI Upper
1	50[c]	50	50	50	50	50
2	50	50	50	50	50	50
3	50	50	50	50	50	50
4	50	50	50	50	50	50
5	1,651	958	2,344	2,086	1,685	2,487
6	2,964	1,998	3,931	3,265	2,100	4,429
7	3,711	3,589	3,833	4,646	4,079	5,213
8	4,559	4,163	4,955	4,856	2,857	6,854
9	12,149	8,205	16,092	10,853	9,478	12,228
10	9,836	5,353	14,319	21,244	14,190	28,299
11	22,087	14,534	29,640	21,244	14,190	28,299

[a]Samples 1–4 are non-immune and 5–11 are immune sera from rhesus macaques vaccinated with either 10 or 20 μg of CP5-CRM^{197} conjugate vaccine. [b]OPA titers are defined as the reciprocal of the highest serum dilution that kills 50% of the bacteria. Each sample was tested in replicates and geometric mean titers with 95% confidence interval were calculated using data from three independent experiments. [c]Negative samples were arbitrarily assigned a titer of 50, which is half of the lowest serum dilution tested in the OPA. [d]Strain PFESA0046 antigen expression levels (MFI): CP5- 9,887; SpA- 633. [e]Strain PFESA0048 antigen expression levels (MFI): CP5–16,400; SpA- 1,205.

Table 4. OPA titers against MSSA strain expressing CP8 antigen

	PFESA0186[d]		
Sample #[a]	GMT[b]	95% CI Lower	95% CI Upper
1	50[c]	50	50
2	50	50	50
3	50	50	50
4	50	50	50
5	13,353	12,311	14,396
6	4,845	4,784	4,906
7	6,973	5,317	8,629
8	11,484	8,970	13,998
9	6,969	5,354	8,584

[a]Samples 1–4 are non-immune and 5–9 are immune sera from rhesus macaques vaccinated with either 20 or 100 μg of CP8-CRM^{197} conjugate vaccine. [b]OPA titers are defined as the reciprocal of the highest serum dilution that kills 50% of the bacteria. Each sample was tested in replicates and geometric mean titers with 95% confidence interval were calculated using data from two independent experiments. [c]Negative samples were arbitrarily assigned a titer of 50, which is half of the lowest serum dilution tested in the OPA. [d]Strain PFESA0186 antigen expression levels (MFI): CP8- 33,300; SpA- 38.

units (CFUs) after 60 min incubation in the presence of complement and HL-60 cells to the number of CFU at time zero (T0). The calculated ratio was subtracted from a value of one and expressed as a percentage. An OPA antibody titer was defined as the reciprocal of the highest serum dilution resulting in 50% reduction of the number of bacterial colonies at 60 min when compared with the background control containing bacteria, complement and HL-60 cells but no serum. Negative samples were assigned OPA titer values of 50, half the value of the first serum dilution in the assay.

OPA specificity experiment. Specificity experiments for OPA were performed as described above with an extra step of adding polysaccharide competitors prior to the opsonization step. In the additional step, either homologous or heterologous staphylococcal polysaccharides were added individually to the test sera at a concentration of 100 μg/ml.

Disclosure of Potential Conflicts of Interest

All authors are employees of Pfizer and as such were paid to do this work and may have shares in the company. J.M.A., J.H. and A.S.A. were previously employed by Merck and may have shares in the company.

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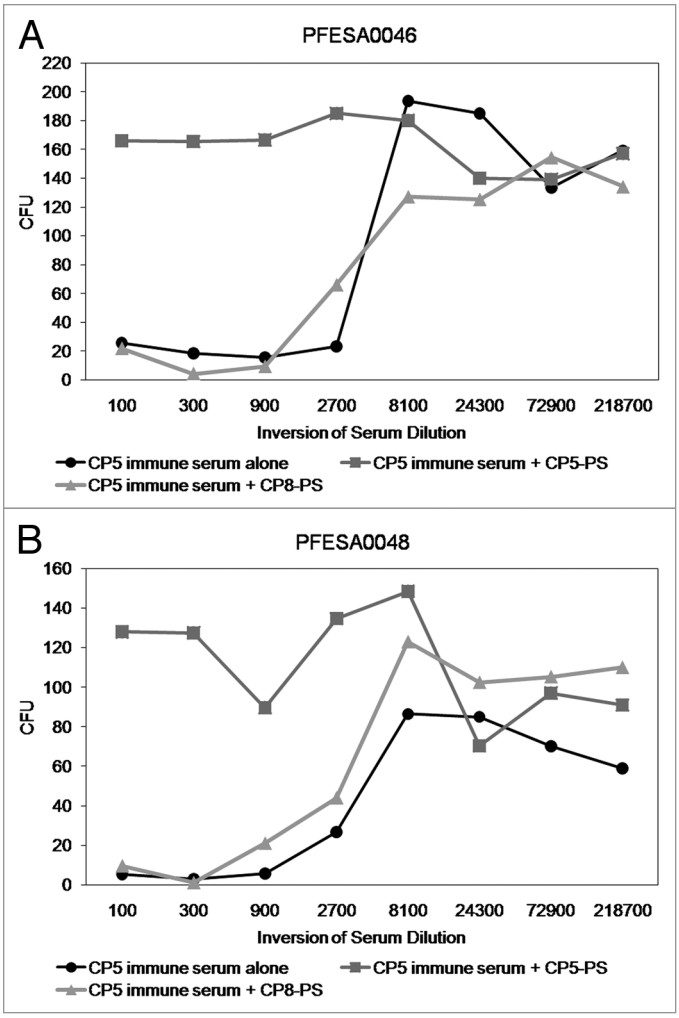

Figure 4. Anti-capsular antibodies show antigen specific killing for MRSA strain PFESA0046 and PFESA0048. CP5 immune rhesus macaque serum OP activity against (**A**) PFESA0046 and (**B**) PFESA0048 strains was competed with 1μg of either purified CP5-PS (homologous polysaccharide) or CP8-PS (heterologous polysaccharide). Abrogation of kill activity in the presence of homologous polysaccharide demonstrates that the assay is specific.

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