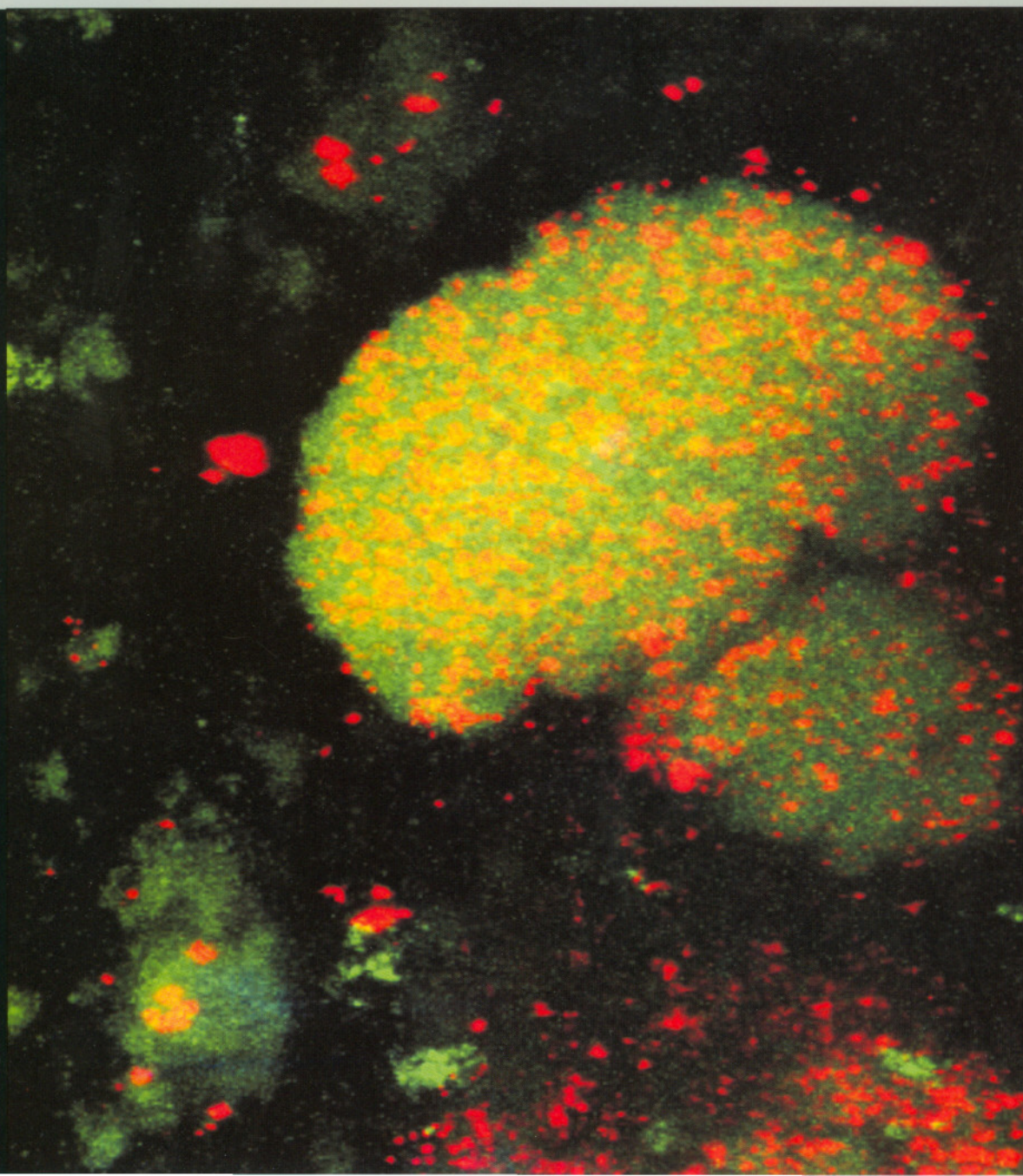


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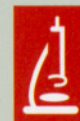


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Immunoglobulins to Surface-Associated Biofilm Immunogens Provide a Novel Means of Visualization of Methicillin-Resistant *Staphylococcus aureus* Biofilms[∇]

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Antigens from the methicillin-resistant *Staphylococcus aureus* (MRSA) cell wall have been shown to be immunogenic in vivo and upregulated during biofilm growth. In this study, we created purified, recombinant forms of selected antigens and biofilm-upregulated, cell wall-associated proteins. These proteins were shown to cause a robust polyclonal immunoglobulin G (IgG) response when used to immunize rabbits. Antibodies against these recombinant proteins bound to the native forms of each protein as harvested from in vitro grown biofilms of MRSA, as determined both via Western blot analysis and immunofluorescence confocal microscopy. These IgGs could be utilized as imaging tools that localize to areas of specific protein production within a biofilm. This work illustrates that immunogenic, cell wall-associated, biofilm-upregulated proteins are promising for in vitro visualization of biofilm growth, architecture, and space-function relationships.

Due to the emergence of multiantibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *S. aureus*, incidences of *S. aureus* infection are becoming more worrisome. A hallmark of a chronic *S. aureus* infection is the bacteria's ability to grow as a biofilm, a sessile community of cells that is attached to a substratum embedded in a matrix of extracellular polymeric substance, and to exhibit altered growth, gene expression, and protein production phenotypes (8). Growth as a biofilm makes eradication of *S. aureus* infections difficult, leading to a persistent, chronic state of disease. Antimicrobial agents and the host immune response are often unable to clear these biofilms. While the mechanisms of antibiotic tolerance in the biofilm are still somewhat unclear, they are thought to be due to altered metabolic activity, diffusion limitation, and differences in the genotypes and phenotypes of biofilm cells compared to planktonic bacteria (2, 9). This facet of growth only complicates treatment of the already resistant MRSA.

Imaging of *S. aureus* biofilms in vitro can lead to new information regarding biofilm architecture and localization-specific protein expression within the biofilm. While methods such as using fluorescent reporter strains (12, 15, 17) and electron microscopy (16) are all valuable for visualizing biofilms grown in vitro, there are limitations associated with each technique. For example, intercalating dyes, fluorescence plasmids, and electron microscopy are all nonfunctional means of visualization that do not give any information about protein expression levels or localization within the biofilm structure. Fluorescent reporter strains are functional indicators of gene expression,

but whether this necessarily indicates protein production and localization is not assured. Moreover, the antibiotics needed to retain a plasmid or to form a stable integrate within a fluorescent strain could alter the phenotype of the biofilm. Confocal laser scanning microscopy (CLSM) is an effective means of visualizing the three-dimensional structure of a biofilm, provided a fluorescent dye is used for visualization (13). Use of CLSM with protein-specific staining that would give functional data as to where and when these proteins are being made would be a significant improvement.

Previous work in our laboratory (5) identified 22 cell wall-associated MRSA proteins that are immunogenic during osteomyelitis infection in the rabbit. In this study, we utilized purified, recombinant forms of several of these immunogens to create polyclonal immunoglobulin G (IgG) against each antigen. These antibodies were then used to probe MRSA biofilms. Since IgG antibodies have been shown to penetrate *Staphylococcus epidermidis* biofilms (6), we hypothesized that antibodies specific to biofilm-upregulated, cell wall-associated antigens within the biofilm may be useful in this respect. In addition, since we utilized antibodies to protein and not poly-*N*-acetylglucosamine (PNAG) (6), we believed that localization would be more specific. The specific binding of these immunoglobulins to the MRSA biofilm allowed for fluorescent visualization via CLSM that shows where and how much of the antigen is made.

MATERIALS AND METHODS

Organisms. MRSA strain MRSA-M2, which was isolated from a patient with osteomyelitis at the University of Texas Medical Branch, and *S. epidermidis* ATCC 35984 were utilized for biofilm growth studies. *Escherichia coli* TOP10 cells were utilized for protein production experiments.

Biofilm growth conditions. MRSA biofilms were grown for all experiments as described in Brady et al. (5). For imaging studies, modification of the silicon tubing was made so that 1-mm square glass tubing (Friedrich and Dimmock, Millville, NJ) was incorporated. *S. epidermidis* biofilms were cultured using the

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TABLE 1. Candidate antigens^a

Antigen characteristic	SA0037	Lipase	SA0688	Glucosaminidase	SA0486
Upregulated during in vitro biofilm growth	+	-	+	+	+
Cell wall associated	+	-	+	+	+
Immunogenic in biofilm infection	+	+	+	+	-

^a Data presented in this table are derived from work performed in the present study and also by Brady et al. (5).

same system as for MRSA, with the exception that a 1:10 dilution of CY broth (containing Casamino Acids and yeast extract) was used without the addition of oxacillin.

Selection of imaging targets. In order to identify biofilm-upregulated genes to pursue as potential imaging targets, microarray analysis was performed comparing biofilm to planktonic growth conditions as described in Brady et al. (5).

Candidate antigens. Proteins that were shown to be immunogenic in our rabbit model of tibial osteomyelitis (5) and/or were found to be cell wall associated by analysis with pSORTb (version 2.0.3) (<http://www.psorb.org/psorb/index.html>), and were also shown to be biofilm upregulated via microarray analysis, were utilized in this work. In addition, we selected one antigen whose cellular localization and gene regulation during biofilm growth led us to believe it would serve well as a negative control. A complete listing of antigens tested is given in Table 1.

Cloning and expression of recombinant antigens. Nucleic acid sequences for each protein were obtained using the GenBank database (<http://www.ncbi.nih.gov/>), and primers were constructed that allowed for amplification of the entire coding region minus the signal sequence (Table 2). In these experiments, two different expression vectors were used: pASK-IBA14 (IBA, Göttingen, Germany) and pBAD-Thio/TOPO (Invitrogen Life Technologies). Primers used for cloning into pASK-IBA14 contained BsaI restriction sites in the 5' ends. The SA0037 gene was cloned into pBAD-Thio/TOPO, as part of the pBAD/TOPO ThioFusion expression system, and transformed into *E. coli* TOP10 cells (Invitrogen Life Technologies) as per the manufacturer's instructions. The other candidate genes were cloned into pASK-IBA14 using BsaI restriction digestion and transformed into *E. coli* TOP10. The clones were grown in Luria broth overnight, diluted 1:50, and grown to exponential phase (*A*₆₀₀ of ~0.5) with shaking (225 rpm). The SA0037 clone was grown at 37°C while the candidates cloned into pASK-IBA14 were cultured at room temperature. A time-zero sam-

ple was taken from each culture, after which exponential phase cultures were supplemented with arabinose (SA0037) at a final concentration of 0.2%. These cultures were allowed to grow for 4 h for induction. Cultures of lipase, glucosaminidase, and the proteins identified as SA0688 and SA0486 were induced by the addition of anhydrotetracycline to a final concentration of 0.2 µg/ml. These cultures were allowed to continue incubating with shaking at room temperature for 3 h as per the manufacturer's directions. Cells were collected by centrifugation at 12,000 × *g*.

Purification of recombinant SA0037. As SA0037 was found to be an insoluble protein (data not shown), we utilized the ProBond Purification System (Invitrogen Life Technologies), as per the manufacturer's instructions, with hybrid purification conditions. The protein was purified using the ProBond purification system's nickel columns, the fractions ("protein-stripped" supernatant, washes, and eluate) were all retained, and samples thereof were resolved on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel to ensure that purification was complete and that all of the recombinant protein was being retained in the eluate (data not shown). Eluted protein was then dialyzed against phosphate-buffered saline (PBS) using Slide-A-Lyzer dialysis membranes with a molecular weight cutoff of 3,500 (Pierce Biotechnology, Rockford, IL).

Purification of recombinant lipase, SA0688, glucosaminidase, and SA0486. Cells were pelleted and lysed through the addition of buffer P (100 mM Tris-HCl [pH 8], 500 mM sucrose, 1 mM EDTA) and incubation on ice for 30 min. A 10-µl sample was removed for analysis to ensure that protein induction was successful. Spheroplasts were removed by centrifugation at 13,000 rpm for 5 min. The supernatant was retained (containing the periplasmic proteins), and a 10-µl sample of the spheroplasts was retained for comparison of the target protein's periplasmic versus cytoplasmic localization. The target protein was then purified using Strep-Tactin spin columns (IBA, Göttingen, Germany) as per the manufacturer's instructions. At each step, 10-µl aliquots were retained for subsequent

TABLE 2. Primers and plasmids used in this study

Primer or plasmid	Sequence (5'-3') or relevant characteristics	Product (size [bp])	Source
Primers			
5' SA0037	ATGAATACAATCAAACTACGAAA	Conserved hypothetical protein (519)	
3' SA0037	CTTCTCATCGTCATCTGATTTCAAAATCCATT TTGA		
5' Lipase	ACTCTAGGTCTCACTCCCATCTGAAACAACATTA TGACCAAAT	Lipase (966)	
3' Lipase	ATGGTAGGTCTCATATCATAAAGGATTTAACGGT AATTCATTACT		
5' SA0688	ATGGTAGGTCTCACTCCGATAAGTCAAATGGCA AACTAAAAGT	ABC transporter, lipoprotein (860)	
3' SA0688	ATGGTAGGTCTCATATCATTTTCATGCTTCCGTGT ACAGTT		
5' Glucosaminidase	ATGGTAGGTCTCACTCCGCTTATACTGTTACTAA ACCACAAAC	Glucosaminidase (1,443)	
3' Glucosaminidase	ATGGTAGGTCTCATATCATTTATATTGTGGGATG TCGAAGTATT		
5' SA0486	ACTCTAGGTCTCACTCAAAGAAGATTCAAAGA AGAACAAAT	Hypothetical lipoprotein (683)	
3' SA0486	ATGGTAGGTCTCATATCAGCTATCTTCATCAGAC GCCCCA		
Plasmids			
pBAD-Thio/TOPO	pUC ori; Amp ^r ; pBAD promoter; for arabinose-inducible expression of PCR product (4,454 bp)		Invitrogen Life Technologies
pASK-IBA14	pUC ori; Amp ^r ; tetA promoter; for tetracycline-inducible expression of PCR product (3,001 bp)		IBA, Göttingen, Germany

SDS-PAGE analysis. Proteins were eluted from the columns via the addition of three 150- μ l volumes of biotin elution buffer (100 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 2 mM D-biotin, pH 8) to ensure maximum protein yield. The eluted proteins were then concentrated approximately 10 times using Centricon centrifugal filters with a molecular weight cutoff of 10,000 (Millipore, Billerica, MA).

Polyclonal IgG production. Purified recombinant antigen (10 μ g) was combined with Titermax Gold adjuvant and mixed via sonication. Each antigen was then injected intramuscularly into 8-week-old female New Zealand White rabbits. Rabbits were bled prior to immunization as a negative control. Booster immunizations were administered two times at 10-day intervals. Ten days after the second boost, animals were bled again. IgG was harvested from the serum via a Melon Gel IgG purification kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions, and IgG was ammonium precipitated overnight. The precipitated IgG was resuspended and dialyzed three times against 1 \times Melon Gel purification buffer. Purified IgG was quantified using the modified method of Bradford (4).

Western blotting. In order to determine if the purified, recombinant proteins were eliciting a robust IgG response upon vaccination, 5 μ g of each protein was resolved on SDS-PAGE gels. The protein was then transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted using the appropriate polyclonal IgG at a 1:100 dilution. Goat anti-rabbit IgG with a horseradish peroxidase tag was utilized as a secondary antibody at a dilution of 1:5,000. Western blots were visualized using a chemiluminescent substrate (SuperSignal; Pierce Biotechnologies).

To analyze the ability of the purified recombinant forms of the proteins to react with serum from animals suffering from MRSA biofilm infections, each protein was resolved and transferred as above, and serum from our animal model of osteomyelitis (5) was used as the primary antibody.

In order to determine if IgG created against the purified recombinant forms of these proteins could effectively bind to their cognate proteins found in the biofilm mode of growth, total biofilm protein as well as cell wall and protoplast fractions were resolved using SDS-PAGE and transferred to PVDF membranes. These membranes were then probed using purified anti-recombinant IgG at a 1:1,000 dilution and goat anti-rabbit IgG-horseradish peroxidase at a 1:5,000 dilution as a secondary antibody, with SuperSignal applied for visualization.

In vitro IgG immunofluorescence experiments. In order to evaluate the ability of the anti-recombinant IgGs to bind to their cognate proteins in their native forms within an intact biofilm, we grew 14-day MRSA or *S. epidermidis* biofilms as described above, with the modification that a flow cell was inserted into the silicon tubing. After 14 days, the tubing on either side of each flow cell was clamped, and the flow cell was excised. The biofilm cells were not fixed or embedded in any way prior to immunofluorescence. The cells were flushed with PBS-3% bovine serum albumin (BSA), and then the polyclonal IgG was injected into the flow cell and incubated at room temperature for 45 min. IgG for each candidate antigen was used in separate experiments; IgG was diluted according to normalization to anti-lipase diluted 1:100 into PBS-1% BSA. The flow cell was flushed by injecting PBS-3% BSA, followed by incubation with a 1:200 (10 μ g/ml) dilution of AlexaFluor 633-conjugated goat anti-rabbit F(ab')₂ (Invitrogen) in the dark for 45 min. The flow cells were again flushed with PBS-3% BSA. SYTO 9 DNA intercalating stain (Invitrogen) was applied at a concentration of 3.34 nM in order to stain all cells within the biofilm, and cells were allowed to incubate in the dark for 15 min. CLSM was employed to visualize the biofilm and binding of the candidate IgG via fluorescence using a Zeiss LSM510 Metalaser scanning confocal microscope. This microscope was not inverted. The microscope was configured with two lasers (argon 488-nm/514-nm/543-nm and HeNe 633-nm lasers), and micrographs were taken at random with the Plan-Apochromat 63 \times oil immersion differential interference contrast optics objective (numerical aperture, 1.4). Filters were set to a band pass of 505 to 530 nm for visualization of SYTO 9 and a long pass of 650 nm for visualization of the conjugated antibody. The sections examined were all approximately 40 μ m thick as determined by the LSMix software (Zeiss).

RESULTS

Immunogenicity of candidate proteins. In previous work (5) we identified several proteins that were immunogenic during biofilm-associated MRSA infection. Other, unpublished work in our laboratory compared the transcriptomic profile of biofilm-associated MRSA to planktonically grown MRSA. From these two experiments, we generated a list of potential imaging targets that met the criteria of being cell wall-associated and

expressed at higher levels within the biofilm. In the work presented herein, we wished to attempt to visualize MRSA biofilms grown in vitro using IgG antibodies specifically targeted to these proteins. Thus, we generated purified, recombinant forms of each protein in order to produce IgG in rabbits.

In order to determine if the epitope structure of the purified recombinant form of each protein matched well with that of the proteins found within the biofilm, an aliquot (5 μ g) of each recombinant protein was resolved via SDS-PAGE, and proteins were transferred to a PVDF membrane. The membrane was then immunoblotted with serum from our rabbit model of osteomyelitis infection (Fig. 1A). All but protein SA0486 robustly reacted with this serum. Therefore, it can be assumed that the recombinant form of the protein is able to be recognized by antibodies directed against the native protein produced during a biofilm infection. With respect to SA0486, this antigen may not elicit a significant antibody response in an in vivo infection due to competition with other antigens. However, due to its high levels of upregulation and its localization to the cell wall, we thought it could still be quite useful as a potential imaging target.

Polyclonal antibody production and analysis. The recombinant proteins were injected into rabbits (10 μ g per injection combined with Titermax Gold adjuvant, with three injections given, each 10 days apart), and serum was collected. Polyclonal antibodies to each protein showed a strong, specific response to both the recombinant protein and the cognate protein from MRSA in vitro biofilms as determined via Western blotting (Fig. 1B). Preimmune serum did not react with the recombinant proteins or total biofilm protein (data not shown). IgG against each recombinant protein was isolated from whole serum via a Melon Gel IgG purification kit (Pierce, Rockford, IL), ammonium precipitated, and dialyzed. When these antibodies were tested against total protein from the cell wall fraction of an in vitro biofilm separated by SDS-PAGE, they bound to proteins that corresponded to the molecular weight of the native protein (Fig. 1C). Therefore, it can be assumed that the recombinant forms of the candidate antigens effectively mimic the in vivo and in situ properties of the native form.

Recombinant SA0486 was not recognized by antibodies directed against the native protein produced during a biofilm infection (Fig. 1A). There are several possible reasons why this occurred. First, there may have been a less than robust immune response to SA0486 in vivo, as this protein may be hidden within the biofilm. However, although an immune response to this antigen may not develop in a biofilm, IgG has been shown previously in our laboratory (data not shown) as well as in work by others (18) to flow freely through the exopolysaccharide matrix. Therefore, this does not prevent the gene product from being used as a potential imaging target. Also, while we saw significantly higher expression of the SA0486 gene in biofilm growth in vitro (via microarray analysis) than in planktonic growth, the expression levels in vivo may not match. Therefore, there may be relatively low levels of SA0486 protein present during infection and, thus, a reduced immune response. Regardless, when we performed the converse study, SA0486 protein as isolated from the biofilm was bound strongly by its anti-recombinant IgG antibody (Fig. 1C). This illustrates that even though this protein was nonimmuno-

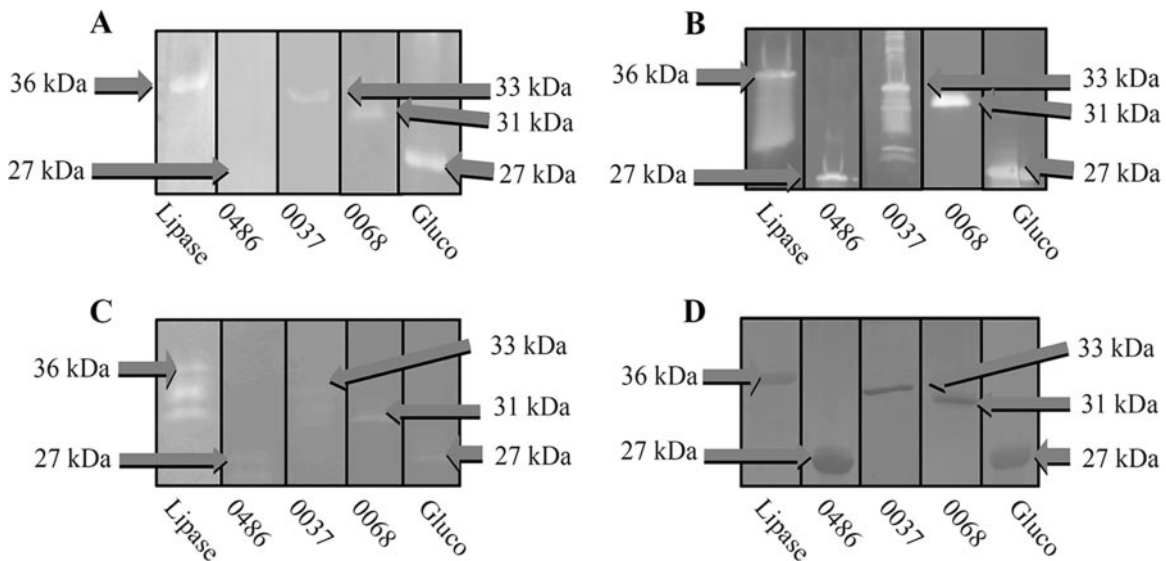


FIG. 1. Purified recombinant proteins elicit a strong antibody response. (A) Purified recombinant proteins were run on an SDS-PAGE gel and probed with convalescent-phase serum from the biofilm infection model. (B) Purified recombinant proteins were run on an SDS-PAGE gel and probed with serum drawn from rabbits vaccinated with individual recombinant proteins. (C) Total protein from the cell wall fraction of an in vitro grown biofilm were run on an SDS-PAGE gel and probed with serum drawn from rabbits immunized with individual recombinant proteins. (D) Coomassie-stained SDS-PAGE gel with 5 μ g of each purified recombinant protein resolved. Arrows point to bands corresponding to the molecular masses of lipase, SA0486, SA0037, SA0688, and glucosaminidase (Gluco), predicted to be 36, 27, 33, 31, and 27 kDa, respectively.

genic in vivo, it is still able to be targeted by anti-SA0486 IgG. The high levels of binding seen also indicate that this protein is present at high levels within the biofilm, at least in vitro.

In vitro visualization of MRSA biofilms using anti-recombinant IgG. We next applied the resulting IgG to a 14-day in vitro-grown *S. aureus* biofilm. An *S. aureus* biofilm was cultured as described by Brady et al. (5), with the modification of using 1-m sections of silicon tubing with square flow cells. The flow cell was flushed, and this step was followed by incubation with specific antibodies and then with AlexaFluor 633 goat anti-rabbit F(ab')₂ (Invitrogen). SYTO 9 DNA intercalating stain was also applied in order to stain all cells within the biofilm. CLSM was employed to visualize the biofilm and binding of the candidate IgG via fluorescence. As is evident in Fig. 2, IgG against proteins that are cell wall-associated and were found, via microarray analysis, to be upregulated in a biofilm (recombinant SA0486, SA0037, glucosaminidase, and SA0688) bound strongly to the intact MRSA biofilm. However, IgG against the gene product that has a low level of secretion into the flowing medium (lipase) did not bind. This illustrates that cell wall-associated proteins that are found at increased levels in the biofilm can be targeted for specific binding by polyclonal IgG. The lack of binding by anti-lipase IgG also demonstrates that the binding by the other IgGs is not due to nonspecific binding to protein A. The lack of reactivity when only secondary antibody was applied (Fig. 2F) also shows that the binding of the antibodies against biofilm-associated antigens is specific.

Specificity of some anti-recombinant IgGs to *S. aureus* biofilms. We also applied the antibodies to *S. epidermidis* biofilms in order to determine the specificity of each IgG to *S. aureus*. While the anti-glucosaminidase, anti-SA0688, and anti-lipase IgGs were unable to bind to *S. epidermidis*, the IgG against the highly conserved SA0486 protein did specifically bind the bio-

film, and anti-SA0037 IgG bound weakly (Fig. 3). This allows us to conclude that anti-glucosaminidase and anti-SA0688 IgGs bind specifically to *S. aureus*. Anti-SA0037 and anti-SA0486 IgGs may be *Staphylococcus* genus specific; future studies will examine this by attempting to utilize these IgGs in the visualization of other staphylococcal species.

DISCUSSION

Diagnosis and treatment of *S. aureus* infections, particularly of multiantibiotic-resistant infections (such as MRSA or vancomycin-resistant *S. aureus*) are difficult. Moreover, visualization of the architecture of these strains in the biofilm mode of growth is restricted. In this work, antibodies against cell wall-associated, biofilm-upregulated antigenic proteins allowed for the visualization of not only the architecture of the *S. aureus* biofilm but also the expression patterns of the target antigens from the observed staining patterns.

The target antigens chosen for this study included one of the two components of autolysin (glucosaminidase) and an uncharacterized ABC transporter lipoprotein (SA0688). *S. aureus* contains cell wall-associated virulence factor protein A. This protein effectively binds to the Fc portion of mammalian IgG as an immunoavoidance strategy. Since the present study is designed to utilize IgG against MRSA biofilm antigens, the IgG-binding ability of protein A may reduce the ability to specifically target certain antigens. Therefore, antibodies against lipase, a secreted antigen that was not significantly upregulated in a biofilm, were developed as a negative control. Two candidates that were previously shown in our laboratory to be cell wall or membrane associated and upregulated under biofilm conditions (unpublished data) were studied for their possible immunogenic potential. These two antigens were not

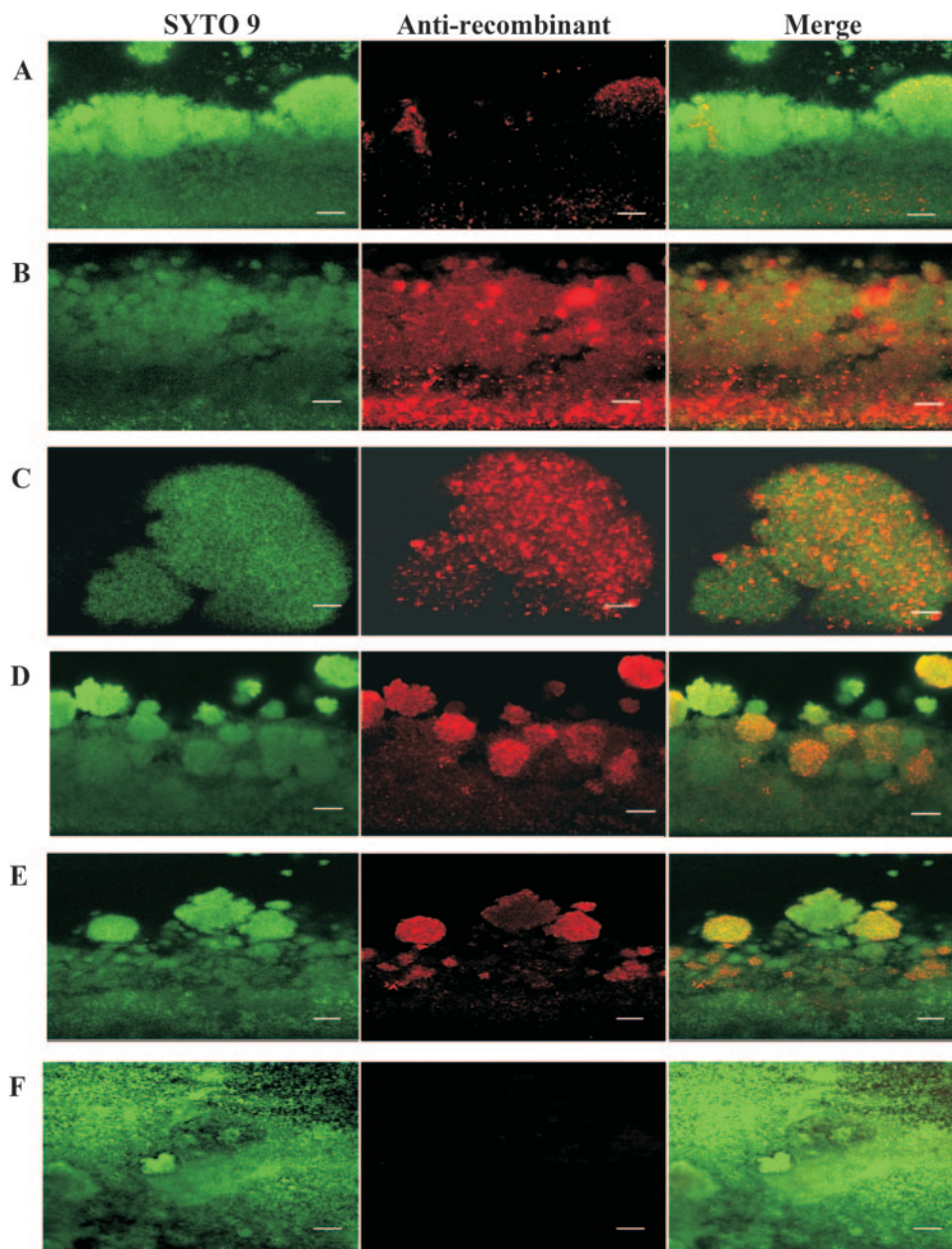


FIG. 2. IgGs against recombinant forms of cell wall-associated biofilm proteins bind to intact MRSA biofilms. MRSA biofilms were grown as described previously (5), and IgG against each selected candidate protein was applied (A to E), followed by the secondary goat anti-rabbit F(ab')₂ antibody (red) (A to F). After a washing step, SYTO 9 was applied to stain all bacterial cells (green). Biofilms were probed with anti-lipase IgG and secondary antibody (A), anti-SA0486 IgG and secondary antibody (B), anti-SA0037 IgG and secondary antibody (D), anti-SA0688 IgG and secondary antibody (D), anti-glucosaminidase IgG and secondary antibody (E), and secondary antibody alone (as a negative control) (F). The base of the glass is located at the bottom of each image, and each image is a cross-sectional view of the biofilm from the base into the lumen. Size bar, 20 μ m.

found in previous screening studies to be immunogenic. However, due to their highly increased transcriptomic levels and their localization to the cell wall, we believed that these proteins could, indeed, be immunogenic and that they were not seen in previous experiments due to shielding by the extracellular matrix, which could lead to a less robust B-cell response. These proteins include SA0037, a conserved hypothetical protein, and SA0486, an uncharacterized lipoprotein. All antigens tested were present in all screened strains.

In order to confirm the similarity of the epitope structure of the recombinant forms of the antigens, as well as to verify the cell wall localization of SA0037 and SA0486, we first undertook a simple Western blot study in which we tested the ability of the recombinant proteins to react with serum from a rabbit model of tibial osteomyelitis. The strong reactivity of the recombinant forms of lipase, SA0688, and glucosaminidase with the convalescent-phase serum confirms the information we had already gained about these antigens in previous work (5). Re-

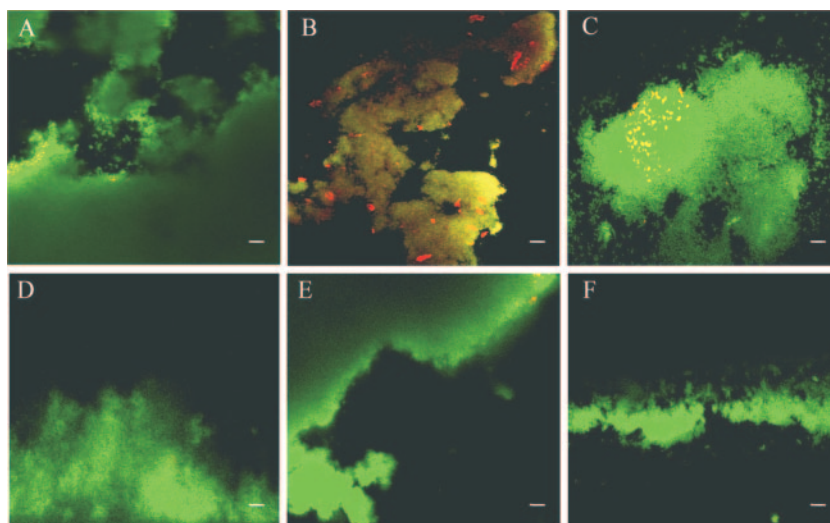


FIG. 3. IgGs against some recombinant *S. aureus* proteins also bind to *S. epidermidis* biofilms. IgGs that show reactivity with the biofilm are seen in red. Total biofilm is shown in green. Goat anti-rabbit F(ab')₂ was used as a secondary antibody. Probes are as follows: anti-lipase IgG and secondary antibody (A), anti-SA0486 IgG and secondary antibody (B), anti-SA0037 IgG and secondary antibody (C), anti-SA0688 IgG and secondary antibody (D), anti-glucosaminidase IgG and secondary antibody (E), and secondary antibody only (F) (as a negative control). Size bar, 20 μ m.

combinant SA0037 was also reactive with this serum, meaning that SA0037 is immunogenic during *S. aureus* biofilm infection, and this indicates that the protein is exposed to the immune response at some point during the infection though protein mapping tools (i.e., pSORT) give an unknown localization.

However, recombinant SA0486 was not reactive with the convalescent-phase sera (Fig. 1A). This means that SA0486, which has a known cell wall association, was not immunogenic during an in vivo infection. This lack of immunogenicity may have been due to the protein's being hidden within the biofilm or masked by another antigen. Nevertheless, SA0486 can still be used as an imaging target since IgG against the recombinant antigen was able to flow freely through the exopolysaccharide matrix and interact with the native form of SA0486 during these in vitro studies.

Although SA0486 transcript levels may have been higher, as shown by earlier microarray studies, this may not necessarily reflect translated products. As a target for a possible imaging tool, this also may not be an issue, as any SA0486 that is present should be bound by the antibody. Regardless, when we performed the converse study, both SA0037 and SA0486 proteins as isolated from the biofilm were bound strongly by their respective anti-recombinant IgG antibodies (Fig. 1C). This shows that in the case of SA0037, its localization is on the outer portion of the cell, which gives us information about its localization that was previously unattainable. For SA0486, these results illustrate that even though this protein was nonimmunogenic in vivo, it is still able to be targeted by anti-SA0486 IgG. The high levels of binding seen also indicate that this protein is present at high levels within the biofilm, at least in vitro. Thus, we hypothesized that SA0486 may still be a worthwhile target for imaging.

In the final part of this work, the ability of the anti-recombinant antibodies to bind to their cognate proteins within an intact, mature *S. aureus* biofilm grown in vitro was monitored.

In these experiments, antibodies generated against purified, recombinant forms of *S. aureus* biofilm proteins bound to those proteins in their native form in an intact biofilm. To our knowledge, this is the first report to show in situ binding to specific cell wall-localized biofilm-associated proteins. Other authors who used immunofluorescence to visualize intact biofilms utilized antibodies against whole bacteria (1, 7, 18) or against polysaccharide matrix (6, 11, 14). Not only does this support the notion that IgG antibodies are able to penetrate *S. aureus* biofilms, but it also shows that these IgGs are not being "held up" in the biofilm by the exopolysaccharide, as suggested by Cerca et al. (6). However, the antibodies used in that study were anti-PNAG IgGs, which would be expected to be bound specifically by PNAG within the biofilm matrix. However, many clinical strains of MRSA do not produce PNAG (10, 19). Thus, it is likely that this mechanism of prevention of opsonophagocytosis by staphylococcal biofilms does not hold true in all cases. More research is needed to determine the specific mechanism by which antibodies against *S. aureus* immunogenic proteins, which we have shown to be able to penetrate these biofilms, are prevented from causing opsonophagocytosis.

This is also the first study that utilized immunofluorescence to give information about the function and localization of the proteins within the biofilm itself. As is evident in Fig. 2, the staining of the *S. aureus* biofilm with each of the reactive IgG antibodies is quite different. Anti-SA0486 antibodies stain the entire biofilm. However, anti-SA0688 and anti-glucosaminidase antibodies stained individual microcolonies within the biofilm, while other microcolonies were not stained at all. Anti-SA0037 IgG stained individual cells within each microcolony, giving a punctate staining pattern. Therefore, the antibodies we used in this study demonstrate that the chosen candidate proteins are being produced in the biofilm and are present on the cellular envelope. In addition, they provide insight into

where their target proteins are being expressed within the biofilm. For example, it is evident that glucosaminidase is produced only in some microcolonies and that its expression is not homogenous throughout the biofilm structure. This protein is part of the autolysin Atl and is involved in peptidoglycan hydrolysis (3). Because peptidoglycan cleavage will occur at high levels within cells that are actively replicating and dividing, it may be that the microcolonies where we see positive staining with anti-glucosaminidase IgG are microcolonies in which the cells are actively dividing. In addition, cellular metabolism may be high in certain microcolonies. Therefore, the specific microcolony staining pattern with the anti-SA0688 IgG may demonstrate that this ABC transporter lipoprotein is expressed in microcolonies that are metabolically active. The extremely punctate staining of anti-SA0037 antibodies is of specific interest. However, we are unable to speculate on the role of SA0037 based on this staining, as there are no known proteins with any homology to it that have a described function.

Finally, we also attempted to visualize the closely related *S. epidermidis* biofilm with the same antibodies in order to test the specificity of our anti-recombinant IgGs. While anti-glucosaminidase and anti-SA0688 IgG did not bind to *S. epidermidis*, anti-SA0037 bound weakly, and anti-SA0486 bound strongly. Thus, we do see specificity of some of our antibodies for *S. aureus* biofilms. Another interesting aspect to the microscopy results shows that homologous proteins from different species may have a high degree of sequence identity but have markedly different epitope presentations. For example, BLASTP shows 61% identity between *S. aureus* and *S. epidermidis* glucosaminidase sequences, and the anti-*S. aureus* glucosaminidase IgG does not bind to *S. epidermidis* biofilms. Conversely, other less closely related proteins have similar epitope presentations, such as is the case with SA0486. Anti-*S. aureus* SA0486 IgG does bind to *S. epidermidis* biofilms, and yet the similarity between the proteins of the two species is only 50%. Thus, the specificity of binding to *S. aureus* versus *S. epidermidis* may have more to do with differences between the species in the temporal expression of these proteins or specific epitopes on the outside of the cells than with sequence identity. These antibodies were also applied to a gram-negative biofilm in order to test specificity to the *Staphylococcus* genus in general. When we utilized *Pseudomonas aeruginosa* in a 14-day biofilm, we saw only relatively weak nonspecific binding of all antibodies, including our secondary F(ab')₂ antibody alone (data not shown), because a small proportion of the antibodies collected in the PAO1 biofilm matrix. Therefore, the fidelity of the IgGs against staphylococcal antigens was demonstrated since they did not interact with homologous proteins in *P. aeruginosa*. Thus, we were able to show that anti-glucosaminidase and anti-SA06988 IgGs are useful to image *S. aureus* while other IgGs are cross-reactive with epitopes expressed in *S. epidermidis*. However, our focus of interest is on *S. aureus* biofilms grown in vitro. This research could be expanded to include antibodies generated against the recombinant forms of *S. epidermidis* proteins to pursue the investigation of those proteins' expression within the biofilm of that species.

Overall, the work presented herein supports the method that recombinant forms of biofilm-upregulated, cell wall- and membrane-associated proteins can be used to create IgG antibodies to be used as imaging tools that are specific to *S. aureus* bio-

films. Additionally, this study also begins to delve into functional research regarding the expression patterns of *S. aureus* biofilm proteins within the biofilm architecture. These data could have useful applications in dissecting the various microcolonies within the entirety of the biofilm, work which would be extremely important in furthering understanding of how these structures form and persist. Lastly, these IgGs may also have great promise for use as in vivo diagnostics; research into utilizing these antibodies in this way is ongoing in our laboratory.

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