# Identification of *Staphylococcus aureus* Proteins Recognized by the Antibody-Mediated Immune Response to a Biofilm Infection

Rebecca A. Brady,[1] Jeff G. Leid,[2] Anne K. Camper,[3] J. William Costerton,[4] and Mark E. Shirtliff[1,5]*

*Department of Microbiology and Immunology, University of Maryland—Baltimore, School of Medicine, 660 W. Redwood Street, Baltimore, Maryland 21201[1]; Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona 86011[2]; Center for Biofilm Engineering, Montana State University, 366 EPS Building, PO Box 173980, Bozeman, Montana 59717[3]; Center for Biofilms, School of Dentistry, University of Southern California, Room 4360 DEN, 925 West 34th Street, Los Angeles, California 90089[4]; and Department of Biomedical Sciences, University of Maryland—Baltimore, Dental School, 666 W. Baltimore Street, Baltimore, Maryland[5]*

*Staphylococcus aureus* **causes persistent, recurrent infections (e.g., osteomyelitis) by forming biofilms. To survey the antibody-mediated immune response and identify those proteins that are immunogenic in an *S. aureus* biofilm infection, the tibias of rabbits were infected with methicillin-resistant *S. aureus* to produce chronic osteomyelitis. Sera were collected prior to infection and at 14, 28, and 42 days postinfection. The sera were used to perform Western blot assays on total protein from biofilm grown in vitro and separated by two-dimensional gel electrophoresis. Those proteins recognized by host antibodies in the harvested sera were identified via matrix-assisted laser desorption ionization–time of flight analysis. Using protein from mechanically disrupted total and fractionated biofilm protein samples, we identified 26 and 22 immunogens, respectively. These included a cell surface-associated β-lactamase, lipoprotein, lipase, autolysin, and an ABC transporter lipoprotein. Studies were also performed using microarray analyses and confirmed the biofilm-specific up-regulation of most of these genes. Therefore, although the biofilm antigens are recognized by the immune system, the biofilm infection can persist. However, these proteins, when delivered as vaccines, may be important in directing the immune system toward an early and effective antibody-mediated response to prevent chronic *S. aureus* infections. Previous works have identified *S. aureus* proteins that are immunogenic during acute infections, such as sepsis. However, this is the first work to identify these immunogens during chronic *S. aureus* biofilm infections and to simultaneously show the global relationship between the antigens expressed during an in vivo infection and the corresponding in vitro transcriptomic and proteomic gene expression levels.**

Up to 20% of patients who undergo surgery acquire at least one nosocomial infection (39); this phenomenon is estimated to add $5 to 10 billion in costs to the U.S. health care system (10, 11). *Staphylococcus aureus* is one of the most common etiologic agents for these infections (6, 52). *S. aureus* is a gram-positive, facultative, anaerobic bacterium that is nonmotile and non-spore forming. *S. aureus* is a normal commensal organism of the human nostrils; approximately 20% of the population are colonized with this bacterium, while 60% of the population are transient carriers (43). *S. aureus* infection can lead to several diseases, ranging from minor skin infections (e.g., furuncles and boils) and eye infections (e.g., keratitis) to serious illnesses including bacteremia, endocarditis, septic arthritis, wound infections, pneumonia, toxic shock syndrome, and osteomyelitis. Incidences of *S. aureus* infection are becoming more worrisome with the emergence of multiple-antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus*.

*S. aureus* possesses several means of immune evasion, including the production of capsular polysaccharides (54, 68, 98, 102), protein A (*spa*) (19, 35, 37, 99), and leukocyte-specific toxins (gamma-hemolysin and Panton-Valentine leukocidin)

(9, 32, 34, 78) and the ability to grow as a biofilm (49, 96). A biofilm is defined as a microbially derived sessile community and typified by cells that are attached to a substratum, interface, or each other, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression, and protein production (24). Biofilm depth can vary from a single cell layer to a thick community of cells surrounded by substantial amounts of polymeric substances. These dense biofilms possess a complex architecture in which microcolonies can exist in distinct pillar- or mushroom-shaped structures (21). An intricate channel network runs throughout the biofilm and functions to provide access to environmental nutrients within even the deepest areas of the biofilm. This mode of growth affords *S. aureus* several advantages over its planktonic counterparts, including the capability of the extracellular matrix to seize and concentrate a number of environmental nutrients (7), prevention of removal by several agents (e.g., antimicrobial agents) and the host immune response (16), and the potential for dispersion via detachment (12). Growth as a biofilm makes eradication of *S. aureus* infections difficult, leading to a persistent, chronic state of disease.

B-cell immunity to *S. aureus* is not well studied. Though previous studies identified *S. aureus* antigens recognized by the antibody-mediated host response during acute infections or from healthy individuals (25, 26, 46, 53, 63, 101, 103), it is unknown what antigens are "seen" by the immune system in the case of biofilm-mediated infections. Elucidation of the

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* Corresponding author. Mailing address: Department of Biomedical Sciences, Dental School, University of Maryland—Baltimore, 666 W. Baltimore Street, Rm. 4-G-11, Baltimore, MD 21201. Phone: (410) 706-2263. Fax: (410) 706-0865. E-mail: mshirtliff@umaryland.edu.

antibody-mediated response would increase understanding of the mechanism(s) by which these infections develop in the face of the host defenses and help to advance novel means of diagnosis and treatment before the infections become chronic. Identification of the repertoire of immunogens is also necessary for effective vaccine design in order to elucidate what proteins are expressed in vivo and present in regions of the biofilm where they are exposed to the immune response.

In this study, we utilized a rabbit model of tibial osteomyelitis and an in vitro biofilm growth system to identify the antigens present during an osteomyelitis infection. By employing two-dimensional (2D) gel electrophoresis (2DGE) and immunoblotting with sera from these infected rabbits followed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis, we were able to identify in vivo-expressed *S. aureus* antigens. The up-regulation of these biofilm antigens was also globally confirmed by microarray analyses. These proteins have great potential for use as vaccines and therapeutics and as targets for novel diagnostic modalities.

## MATERIALS AND METHODS

**Organism and reagents.** The strain of *Staphylococcus aureus* used in this study was obtained from a patient with osteomyelitis who was undergoing treatment at The University of Texas Medical Branch, Galveston, Texas. The strain is MRSA and denoted MRSA-M2. Urea, thiourea, β-glycerophosphate, oxacillin, trichloroacetic acid, raffinose, lysostaphin, iodoacetamide, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Aldrich Chemical Inc., St. Louis, MO. Immobiline DryStrips (pH 4 to 7 or 3 to 10 [linear]), Pharmalytes (pH 3 to 9), dithiothreitol (DTT), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), the Multiphor II isoelectric focuser, and a Hoefer DALT vertical system were obtained from Amersham Biosciences (Piscataway, NJ). Most other chemicals and media, including glucose, yeast extract, NaCl, Tris base, and MgCl$_2$, were obtained from Fisher Scientific Inc.

**Growth of *S. aureus* biofilm in vitro.** Because attempts to isolate purified bacterial RNA or protein from bone infected in vivo with *S. aureus* have not been successful (data not shown), an in vitro flow reactor system was used for reproducible biofilm growth. The reactor system (Fig. 1) was constructed within a 37°C incubator and consisted of silicone tubing through which a 1:10 dilution of CY broth (10 g Casamino Acids, 10 g yeast, 5 g glucose, 5.9 g NaCl, 6 mM β-glycerophosphate, 400 mg oxacillin per liter) flowed in a once-through fashion to a waste container under the control of a peristaltic pump. Prior to inoculation, medium was pumped through the system and allowed to equilibrate to temperature for 24 h. An overnight culture of *S. aureus* grown in 1× CY broth was diluted 1:100 into prewarmed 1× CY broth and allowed to grow at 37°C with shaking (225 rpm) until exponential phase was reached. The tubing was clamped upstream of the injection port, and 5-ml portions of the exponential-phase bacterial culture were injected into each silicone tube. The system was allowed to incubate without flow for 30 min so that the bacteria could adhere to the internal surfaces of the tubing. The media flow was restored at a flow rate of 0.7 ml/min, providing a 7.5-min residence time at 37°C. The biofilm was grown on the tubing with this flow rate for 14 days. At day 14, the biofilm was harvested by squeezing the biofilm from the tubing into protein preservation solution (2.8 mM PMSF, 10 mM Tris-Cl, and 1 mM EDTA, pH 8.0) in order to prevent protein expression changes or degradation. Total protein was collected from the biofilm by mechanical disruption using a FastPrep instrument (Q-Bio Gene, Irvine, CA) and 0.1-mm-diameter silica beads and quantified using a modification of the method of Bradford (13).

**Fractionation.** Preliminary studies showed that the total protein preparation described above provided mostly cytosolic proteins. Therefore, we fractionated the biofilm into cytosolic, membrane, and cell wall fractions. The lysostaphin digestion protocol we used to isolate cell wall-associated proteins is well documented (51, 65, 79, 84, 101) and was as described in the work of Nandakumar et al. (65). Briefly, harvested biofilm was centrifuged to collect the bacteria, and the pellet was resuspended in 5 ml lysis buffer (50 mM Tris-Cl, 20 mM MgCl$_2$, pH 7.5) supplemented with 30% raffinose (Sigma, St. Louis, MO). PMSF and lysostaphin (40 μg; Sigma, St. Louis, MO) were added. The suspension was incubated at 37°C without shaking for 35 min and then centrifuged at 6,000 × g at 4°C for 20 min. The supernatant (~5 ml) was collected and contained the cell wall
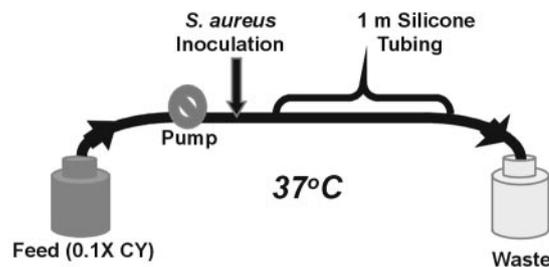


FIG. 1. In vitro biofilm reactor system. For a detailed description, please refer to Materials and Methods.

protein fraction. The pellet was resuspended in 1.0 ml lysis buffer, and then 0.1-mm-diameter silica beads (0.7 g) were added to the cell suspension and disruption was done with a FastPrep instrument. Disrupted cells were then centrifuged at 50,000 × g for 60 min, and the resulting supernatant was isolated. This supernatant contained the cytoplasmic protein fraction, while the pellet contained the cell membrane fraction. The membrane fraction was resuspended in 1.0 ml lysis buffer. At this point, the fractions were ready for trichloroacetic acid precipitation and 2D separation as outlined below.

**2DGE.** Two-dimensional electrophoresis was conducted according to the principles of O'Farrell (71) and as outlined by Gorg et al. (36) and Sauer and Camper (87). To accomplish rehydration of the protein, 500 μg of crude protein was extracted by adding a 1/10 volume of an ice-cold 1:10 mixture of trichloroacetic acid (Sigma, St. Louis, MO)-acetone. The resulting pellet was then directly solubilized in rehydration buffer (0.1 mM urea, 25 μM thiourea, 0.35 μM DTT, 0.5% [wt/vol] CHAPS, and 1.6% Pharmalyte [pH 3 to 10]). These samples were applied to 18-cm Immobiline DryStrips, pH 3 to 10 or 4 to 7 (linear) (GE Healthcare, Piscataway, NJ). The isoelectric focusing, which separated the proteins based on their pIs, was performed using a Multiphor II focuser from Amersham per the manufacturer's directions. Prior to the second dimension, the IPG strips were equilibrated (per the manufacturer's directions) and subsequently applied to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gels. For the resolution of *S. aureus* crude protein extracts in the second dimension, the 26- by 20-cm 2D gel system from the Hoefer DALT vertical system (Amersham Biosciences, Piscataway, NJ) was used. Crude protein extracts were separated at 10°C on an 11% resolving gel, which was then nondestructively silver stained (33).

**Production of osteomyelitis.** The bacterium was grown overnight in tryptic soy broth supplemented with oxacillin (40 μg/ml) and diluted in saline to a concentration of 1.0 × 10$^6$ CFU per ml. Three New Zealand White female rabbits, 8 weeks of age and weighing 1.5 to 3.0 kg, were used. All procedures were performed per humane criteria set forth by the University of Maryland Baltimore Animal Care and Use Committee. Rabbits were anesthetized using an intramuscular injection of 30 mg ketamine (Ketaset; Fort Dodge Laboratories, Inc., Fort Dodge, Iowa)/kg of body weight, 10 mg/kg acepromazine (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa), and 1 mg/kg xylazine (Rugby Laboratories, Inc., Rockville Center, NY). An 18-gauge needle was inserted percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity. Sodium morrhuate (Eli Lilly, Indianapolis, Indiana) (0.1 ml of a 5% [wt/vol] solution), 0.1 ml of *S. aureus* (1.0 × 10$^6$ CFU), and 0.2 ml of sterile saline were injected sequentially (55–58, 69). The needle was removed and the rabbits were returned to their cages. The infection was allowed to progress for 42 days, with sera being drawn at days 0, 14, 28, and 42. While this model of osteomyelitis requires a large organism inoculation, it produces clinical manifestations like those seen in cases of human chronic osteomyelitis, including disruption of the normal bone architecture and periosteal elevation. Also produced is the hallmark of chronic osteomyelitis, the involucrum, which is live, encasing bone that surrounds infected dead bone within a compromised soft tissue envelope (75). In addition, the recalcitrance to clearance by antimicrobial agents and the host immune system that is mediated by a biofilm mode of growth is evident after 28 days of infection.

**Bone cultures.** At the conclusion of the study, rabbits were sacrificed by an intravenous injection of sodium pentobarbital. Both tibias were removed, dissected free of all soft tissue, and processed for bacterial cultures. By use of a 5.0-mm, single-action rongeur, the bones were split into small pieces and the marrow was removed. The whole bone was then pulverized and suspended in 3 ml of sterile 0.85% saline per gram of bone. The marrow was placed in 10 ml of sterile 0.85% saline per gram of marrow. Serial 10-fold dilutions were performed,

and the solution was streaked onto a tryptic soy agar blood plate supplemented with oxacillin (40 µg/ml) to confirm the presence of *S. aureus* in the bone tissue.

**Western blotting.** Bacterial proteins separated by 2D gel electrophoresis were transferred to nitrocellulose and blocked in 5% nonfat dry milk in Tris-buffered saline–Tween 20, pH 7.6 (TBS-T) (20 mM Tris, 137 mM NaCl, 0.1% Tween 20), overnight and then washed three times for 20 min in TBS-T at 25°C. The membrane was treated with convalescent-phase rabbit sera (diluted 1:10) in TBS-T for 60 min. Membranes were washed three times as described above. Goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used at a dilution of 1:5,000 for 60 min in TBS-T, followed by three washes as described above. Immunogenic proteins were detected using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) as described by the manufacturer. Enhanced chemiluminescence-treated membranes were then imaged using a FluorChem 8800 imaging system and the associated imaging software (AlphaEaseFC). Western blotting (85) was performed in triplicate with sera from each of the three infected rabbits. Images of the 2D gel electrophoresis gels and the enhanced chemiluminescence-treated membranes were compared to identify immunogenic protein spots. Immunogenic spots appearing on at least two out of three gels were excised and identified using MALDI-TOF analysis.

**MALDI-TOF analysis.** For identification of immunogenic proteins, an Applied Biosystems Voyager-DE STR MALDI-TOF mass spectrometer was used operated in the positive-ion mode with α-cyano-4-hydroxycinnamic acid matrix for ionization. At least 100 laser shots per spectrum were averaged. Mass spectral peaks with a signal-to-noise ratio greater than 5:1 were deisotoped, and the resulting monoisotopic masses were used for protein identification performed by use of mass fingerprint analysis. The software used for protein identification was the Profound search engine using the Genomic Solution's Knexus software (version 2004.03.15), and the database used was the latest NCBI nonredundant database obtained from NIH. Proteins with an expectation score of $1 \times 10^{-3}$ or lower were considered positive identities.

**Sampling conditions to prevent RNA (for microarray studies) expression profile changes or degradation.** In order to obtain RNA samples from day 14 biofilms, the biofilms were scraped into cold RNAlater (Ambion, Inc., Austin, TX), and the surfaces were then flushed with more cold RNAlater. The resulting bacterial suspension was centrifuged at $12,000 \times g$ for 5 min at 4°C. The cell pellet was resuspended in RNAlater and spun at $12,000 \times g$ for 3 min at 4°C, and the supernatant discarded. Total RNA was then immediately isolated by use of a QIAGEN RNeasy Protect kit (QIAGEN Inc., Valencia, CA). For in vitro planktonic cultures, aliquots were obtained at early logarithmic, late logarithmic, and stationary growth phases (as determined by growth curves), and CFU counts were determined by serial dilution and plating. For microarray experiments, planktonic cultures were diluted in equal volumes of RNAlater (Ambion Inc.). The resulting bacterial suspension was centrifuged at $12,000 \times g$ for 5 min at 4°C. The cell pellet was resuspended in RNAlater and centrifuged at $12,000 \times g$ for 3 min at 4°C, and the supernatant was discarded. Total RNA was then immediately isolated as discussed below.

**RNA isolation techniques.** Total RNA was isolated using the QIAGEN RNeasy Protect (QIAGEN Inc.) protocol with the following modifications. Briefly, *S. aureus* bacterial pellets were resuspended in a highly denaturing buffer containing GITC (buffer RLT [supplied with the kit] supplemented with 10 µl β-mercaptoethanol/ml). Cells were disrupted via a mechanical disruptor (Bio-Spec Products, Bartlesville, OK) run at maximum speed for 2 min and subsequently centrifuged (5 min, $14,000 \times g$). The supernatant was transferred to an RNase-free microfuge tube, ethanol was added, and the supernatant was then applied to a silica gel spin column to bind the RNA. The column was washed several times with RPE (supplied), and the RNA was eluted with diethyl pyrocarbonate-treated water (0.1% diethyl pyrocarbonate). RNA concentration and purity were determined by spectrophotometry. Purified RNA was DNase I treated, and recombinant RNAsin (RNase inhibitor; Promega, Inc., Madison, WI) (10 units) and dithiothreitol (to a final concentration of 10 mM) (Amersham Biosciences, Piscataway, NJ) were added to each sample of purified RNA before samples were stored at −70°C until use. Total RNA quality was confirmed by combining RNA (10 µg) from each *S. aureus* growth condition in a microfuge tube with an equal volume of Glyoxal sample loading dye (Ambion Inc.) to a final volume of 20 µl. After being incubated for 30 min at 50°C, the tubes were spun and placed on ice. Cooled glyoxylated RNA samples were then loaded in duplicate onto a Reliant RNA gel (BioWhittaker Molecular Applications, Inc.) with 1× MOPS (morpholinepropanesulfonic acid) running buffer (Ambion Inc.). The gel was electrophoresed at 35 V, and the quality of RNA bands (16S and 23S) and lack of smear patterns reflecting digested DNA or RNA were determined. For each experiment, only 10 µg total RNA was required for probing of the DNA genomic array.

**Preparation of labeled *S. aureus* cDNA.** RNA (10 µg) from each evaluated culture condition was individually combined with 2 µl of random hexamers (3 mg/ml) (Invitrogen Life Technologies, Carlsbad, CA) and nuclease-free water (Sigma-Genosys, St. Louis, MO) up to a final volume of 17 µl. The RNA and primer combination was incubated at 70°C for 10 min. This was followed by a snap-freeze in a dry ice/ethanol bath for 30 seconds and centrifugation at >10,000 × g for 1 min at room temperature. A 50× aminoallyl-dNTP mix was made with dNTPs (dATP [25 mM], dCTP [25 mM], dGTP [25 mM], and dTTP [15 mM]; Invitrogen Life Technologies) and aminoallyl-dUTP (10 mM; Sigma Chemical Co., St. Louis, MO). The reverse transcription reaction mix (5× first-strand buffer [6 µl], 100 mM DTT [3 µl], 50× aminoallyl-dNTP mix [0.6 µl], 20 units/µl SUPERase●In RNase inhibitor [1.5 µl], and 200 units/µl SuperScript II [2 µl]; Invitrogen Life Technologies) was combined in a separate nuclease-free microfuge tube and added to the denatured, cooled RNA-primer mixture. The reaction mixture was incubated at 42°C overnight. The reverse transcriptase was inactivated at 70°C for 10 min and then chilled on ice. RNA was eliminated by the addition of 10 µl of 1 M NaOH and 10 µl of 0.5 M EDTA (Ambion Inc.) and subsequent incubation at 65°C for 15 min. To neutralize the high pH, 10 µl of 1 M Tris (Ambion Inc.) was added. The resulting cDNA was purified with a QIAquick PCR purification kit per the manufacturer's protocols with the following modifications. The QIAGEN wash and elution buffers were replaced by a phosphate wash buffer (potassium phosphate [5 mM], pH 8.0, 80% ethanol) and a phosphate elution buffer (potassium phosphate [4 mM], pH 8.5) because the QIAGEN buffers contain free amines which compete with the Cy dye-coupling reaction. Following two elutions from the QIAquick column with 30 µl of phosphate elution buffer per elution, the purified cDNA was dried (via speed vacuum) and resuspended in 4.5 µl of fresh 0.1 M $Na_2CO_3$, pH 9.0 (adjusted with concentrated $HCl_{aq}$). Cyanine dye esters (Amersham Biosciences) were prepared by resuspending a tube of dried Cy3 or Cy5 dye ester in 73 µl of dimethyl sulfoxide (Sigma Chemical Co.). An aliquot (4.5 µl) of the appropriate dimethyl sulfoxide-diluted Cy dye ester was added to the purified and resuspended cDNA and incubated at room temperature in the dark for 1 hour. The reaction mixture was purified with a QIAquick PCR purification kit with the modifications described above and eluted in a total of 60 µl of diphosphate elution buffer. To determine the efficiency of the labeling reaction, the entire undiluted eluate for each sample was utilized in spectrophotometry at 260 nm and either 550 nm for Cy3 or 650 nm for Cy5 as appropriate. The number of picomoles of dye incorporation per sample and the nucleotide/dye ratio were determined. Samples that had >200 picomoles of incorporated dye and a ratio of less than 50 nucleotides/dye molecule were optimal for hybridizations. After analysis, the two differentially labeled probes were mixed together (Cy3 versus Cy5) and dried via speed vacuum.

**Microarray development and construction.** DNA microarrays were constructed and made available by The Institute for Genomic Research, Pathogen Functional Genomics Resource Center (PFGRC), through a grant by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (N01-AI-15447). The genome microarray consists of PCR products representing segments of 2,576 open reading frames from *S. aureus* reference strain COL, as well as 117 unique open reading frames from strains Mu50 (59), MW2 (50), and N315 (5) which are not present in the COL strain's genome complement. The targets were printed in triplicate on the array.

**Hybridization conditions.** The staphylococcal genomic DNA microarrays were prehybridized for 45 min at 42°C in filter-sterilized prehybridization buffer (5× SSC [Ambion Inc.] [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS [Ambion Inc.], 1% bovine serum albumin [Sigma-Genosys]) that had been prewarmed to 42°C for 30 min. The microarray slide was then washed twice with double-distilled $H_2O$ and once with isopropanol and air dried. The dried, labeled probe (see above) was resuspended in 22 µl of 1× hybridization solution (50% formamide [Sigma-Genosys], 5× SSC, and 0.1% SDS), and, in order to block nonspecific hybridization, 4 µl of sheared salmon sperm DNA (10 mg/ml; Ambion Inc.) was added. The mixture was heat denatured at 95°C for 3 min, snap-chilled on ice for 30 seconds, and centrifuged at >10,000 × g for 1 min. The prehybridized microarray slide was placed in the hybridization chamber and the denatured probe mixture was added to the array. The array was covered with a cleaned 22- by 60-mm microscope glass coverslip, bubbles were eliminated, 10 µl of water was placed in the wells at each end of the chamber, and the chamber was sealed. The apparatus was then wrapped in light-tight foil and incubated in a 42°C water bath for 20 h. After incubation, the microarray slide was removed from the hybridization chamber in low-light surroundings, submerged in a dish containing prewarmed (42°C) low-stringency wash buffer (1× SSC and 0.2% SDS), and agitated for 4 min to remove the coverslip. The slide was transferred to a dish containing high-stringency buffer (0.1× SSC and 0.2% SDS) and washed with agitation for 4
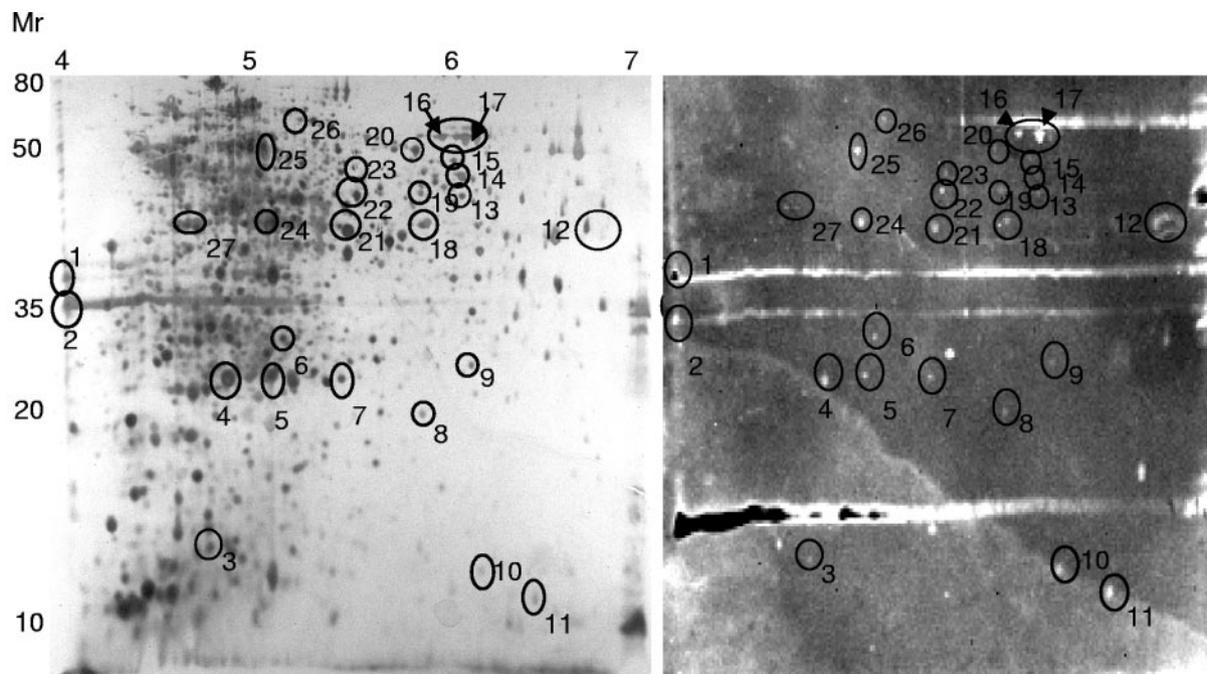
FIG. 2. A representative 2DGE gel and Western blot of mechanically disrupted protein from a day 14 *S. aureus* biofilm showing 27 immunogenic protein spots (circled). pIs are indicated at the top.

min. The slide was given a final wash in 0.1× SSC for 4 min and two rinses in deionized water, air dried, and stored in a light-tight slide box until scanning.

**Analysis and quantitation.** Hybridized microarrays were read utilizing the ScanArray Lite microarray analysis system (Packard Biochip Technologies, Billerica, MA), and the spot intensities were determined, normalized, and evaluated using The Institute for Genomic Research programs Spotfinder, MIDAS, and MultiExperiment Viewer. Triplicate arrays were read and compared in order to ensure accurate and reliable scientific technique. Those spots showing statistically significant ($P < 0.05$) up- or down-regulation (1.5-fold difference) were visually confirmed in each of the three replicates.

## RESULTS

**Visualization of immunogenic proteins.** We began this investigation using simple mechanical disruption to extract proteins from *S. aureus* biofilm samples as a preliminary screen to determine if Western blotting on 2DGE would allow us to visualize a significant number of immunogens. Mechanically disrupted protein from the in vitro day 14 biofilm was visualized by separation in the first dimension in a pH range of 4 to 7 as the characteristic two-dimensional protein pattern for *S. aureus* features a large conglomeration of proteins with high molecular weights and pIs of 4 to 6. Western blotting on these gels was performed using sera collected 28 days postinoculation from rabbits that were infected intratibially with MRSA-M2. This led to the detection of 27 immunogenic protein spots (Fig. 2). The spots were characterized through MALDI-TOF analysis, leading to 24 positive identities (Table 1). While previous studies showed that mechanical disruption of bacterial cells for total protein extraction mainly isolated cytoplasmic proteins, a number of membrane-bound, cell wall-anchored, and synthesized secreted proteins were also found (87, 88). This agreed with our findings in that the majority of the identified proteins from the total cellular samples were also cytoplasmic.

We furthered our studies by use of fractionated proteins

from the biofilm samples, since the cell wall and membrane fractions should contain the most immunologically relevant proteins. 2DGE and Western analyses showed that antigenic proteins were located in all three cellular fractions (Fig. 3). MALDI-TOF analysis was performed on immunogenic protein spots that were excised from two-dimensional gels of the cell wall fraction, and in total 22 proteins were identified (Table 2). Upon receiving protein identities, we obtained the sequence for each protein from Entrez (www.ncbi.nih.gov) and used pSORTb version 2.0 (www.psort.org/psortb/index.html) in order to determine where the protein was located within the cell. Only three of these proteins were found by pSORT or literature searches to be strictly cytoplasmic, further illustrating the efficacy of our cell wall isolation protocol.

Lipase and autolysin, two extracellular proteins, and an ABC transporter lipoprotein involved in extracellular binding were identified from several of the spots. Lipase functions in virulence by degrading lipids in order to help the bacterium acquire nutrients (100), while autolysin aids in cell division by degrading peptidoglycan (73). The aforementioned lipoprotein, superoxide dismutase, elongation factor Tu, and serine hydroxymethyl transferase, as well as alkyl hydroperoxide reductase (subunit C), were identified from this fraction as well as from the whole-cell two-dimensional gel, as discussed above and shown in Table 1. Phosphoglycerate mutase, which is important in the glycolysis pathway, was discovered. We also identified the alpha-hemolysin precursor as an immunogen in the cell wall fraction. This protein is a well-known virulence factor. As mentioned, only a few of the detected proteins in the cell wall fractions were predicted as being cytoplasmic, but these proteins have been shown to be localized in the cell wall in other studies (31, 40, 77).

TABLE 1. Identities of immunogens from total cellular protein[a]

| Spot(s) | Mol wt | pI | Identity | Accession no. | Localization | Function |
|---|---|---|---|---|---|---|
| 1 | 34.78 | 8.7 | Lipoprotein | 15923621 | Extracellular | Unknown |
| 2 | 31.39 | 9.6 | β-Lactamase | 10956176 | Extracellular | Inactivation of β-lactams (42) |
| 3 | 15.44 | 4.7 | Hypothetical protein SAV0828 | 15923818 | Unknown | Unknown |
| 4 | 21.13 | 4.9 | Alkyl hydroperoxide reductase subunit C | 15923371 | Extracellular, cytosol | Reduction of reactive oxygen intermediates (18) |
| 5, 7 | 22.69 | 5.1 | Superoxide dismutase | 15924543 | Extracellular | Conversion of $O_2^-$ to $H_2O_2$ and $O_2$ (41) |
| 6 | 25.55 | 5.3 | ScdA | 1575061 | Cytosol | Peptidoglycan metabolism and cell development (17) |
| 8 | 18.51 | 5.6 | Conserved hypothetical protein | 15924700 | Extracellular | Unknown but similar to that of universal stress protein[b] |
| 9 | 20.60 | 5.7 | Formylmethionine deformylase homolog | 21282703 | Cytosol | Formylmethionine→methionine (14) |
| 10, 11 | 43.14 | 4.7 | Elongation factor Tu | 15923538 | Extracellular, cytosol | Carrier of amino acid/tRNA to the ribosome in translation (1) |
| 12 | 37.34 | 6.1 | IlvA | 21283056 | Cytosol | Threonine deaminase (90) |
| 13 | 40.09 | 5.6 | Alanine dehydrogenase | 21283381 | Cytosol | L-Alanine→pyruvate (91) |
| 14 | 45.33 | 5.8 | Serine hydroxymethyl transferase | 15925103 | Extracellular, cytosol | Synthesis of serine[c] |
| 15 | 54.22 | 5.7 | Probable cytosol aminopeptidase | 15923932 | Cytosol | Removal of amino acids from N or C terminus of peptides (50) |
| 16, 17 | 60.01 | 5.7 | Formyltetrahydrofolate synthetase | 21283404 | Cytosol | Addition of formate to tetrahydrofolate (62) |
| 18 | 35.11 | 5.6 | 6-Phosphofructokinase | 32129696 | Extracellular, cytosol | ATP + D-fructose 6-phosphate = ADP + D-fructose 1,6-bisphosphate[c] |
| 19 | 46.72 | 4.9 | Dihydrolipoamide succinyltransferase | 15924402 | Cytosol | Production of succinyl-CoA (44) |
| 20 | 52.92 | 5.6 | Inositol-monophosphate dehydrogenase | 21282095 | Cytosol | Purine metabolism[c] |
| 21 | 36.43 | 5.3 | Alcohol dehydrogenase | 21282297 | Cytosol | Fermentation (4) |
| 22 | 44.37 | 5.4 | Hypothetical protein SAV0941 | 15923931 | Cytosol | Unknown |
| 23 | 49.37 | 5.3 | CoA disulfide reductase | 21282581 | Cytosol | Dimeric flavoprotein that catalyzes the NADPH-dependent reduction of oxidized CoA (23) |
| 24 | 32.59 | 5.2 | Elongation factor Ts | 15924247 | Extracellular, cytosol | Removal of GDP from EF-Tu–GDP complex (67) |
| 25 | 53.67 | 5.0 | Aspartyl/glutamyl-tRNA amidotransferase subunit B | 20454935 | Unknown | Catalyzes formation of Asn-tRNA and/or Gln-tRNA[b] |
| 26 | 34.22 | 4.7 | Manganese-dependent inorganic pyrophosphatase | 15924909 | Unknown | Removal of pyrophosphate created by many biosynthetic pathways (104) |
| 27 | 35.23 | 4.6 | Pyruvate dehydrogenase E1 component beta subunit | 15924084 | Cytosol | Pyruvate→acetyl-CoA + $CO_2$[c] |

[a] CoA, coenzyme A. Spots are those indicated in Fig. 2.
[b] Protein functions were found on the Entrez database (www.ncbi.nih.gov).
[c] Protein functions were found on the Expasy server (www.expasy.org).

**Evaluation of transcriptional profile for identified immunogens.** Previous studies have looked at a "snapshot" of *S. aureus* biofilm versus planktonic gene regulation using only one or a few time points (4, 5, 81). However, biofilm studies with other bacterial species have shown that each biofilm stage of maturation is significantly different in gene expression and protein production to such a degree that planktonic samples are often transcriptionally and proteomically closer to some biofilm stages than the stages are to one another. Therefore, the comparison between the immunogens we found up-regulated in an in vivo biofilm infection and the proteins found up-regulated in these previous studies may be inappropriate and lacking. For a more thorough comparison between the immunogens detected in our study and the transcription profiles of these genes in an in vitro biofilm, we wished to observe the up- or down-regulation of these particular immunogens over a wider range of time points that included early exponential (2 h), late exponential (6 h), and stationary (48 h) phases of planktonic growth, each compared to early (8 h), maturing (48 h), and fully mature (336 h) biofilm. As is evident in Table 3, the majority of those proteins deemed immunogenic in the cell wall fraction of the *S.*

*aureus* biofilm (Table 2) were up-regulated under biofilm conditions during at least one growth stage.

**Variation in antibody responses over time.** In order to determine if the antibody-mediated response changed as the *S. aureus* osteomyelitis infection progressed, we compared Western blots of cell wall fractions from day 14 in vitro-grown *S. aureus* biofilm probed with sera from uninfected rabbits as well as from rabbits 14, 28, and 42 days postinoculation. No protein spots were immunogenic in the uninfected (day 0) rabbits (Fig. 4). Lipase, autolysin, superoxide dismutase, and a lipoprotein were found to be immunogenic at days 14, 28, and 42 postinoculation, while other isoforms of lipase and autolysin were immunogenic only after 28 days. Other proteins, such as elongation factor Tu and transketolase, were immunogenic only after 42 days of infection (Table 4).

## DISCUSSION

Although biofilms are intrinsically resistant to the host response, little is known about the immune reaction to *S. aureus* in biofilm infections. To study the antibody-mediated immune
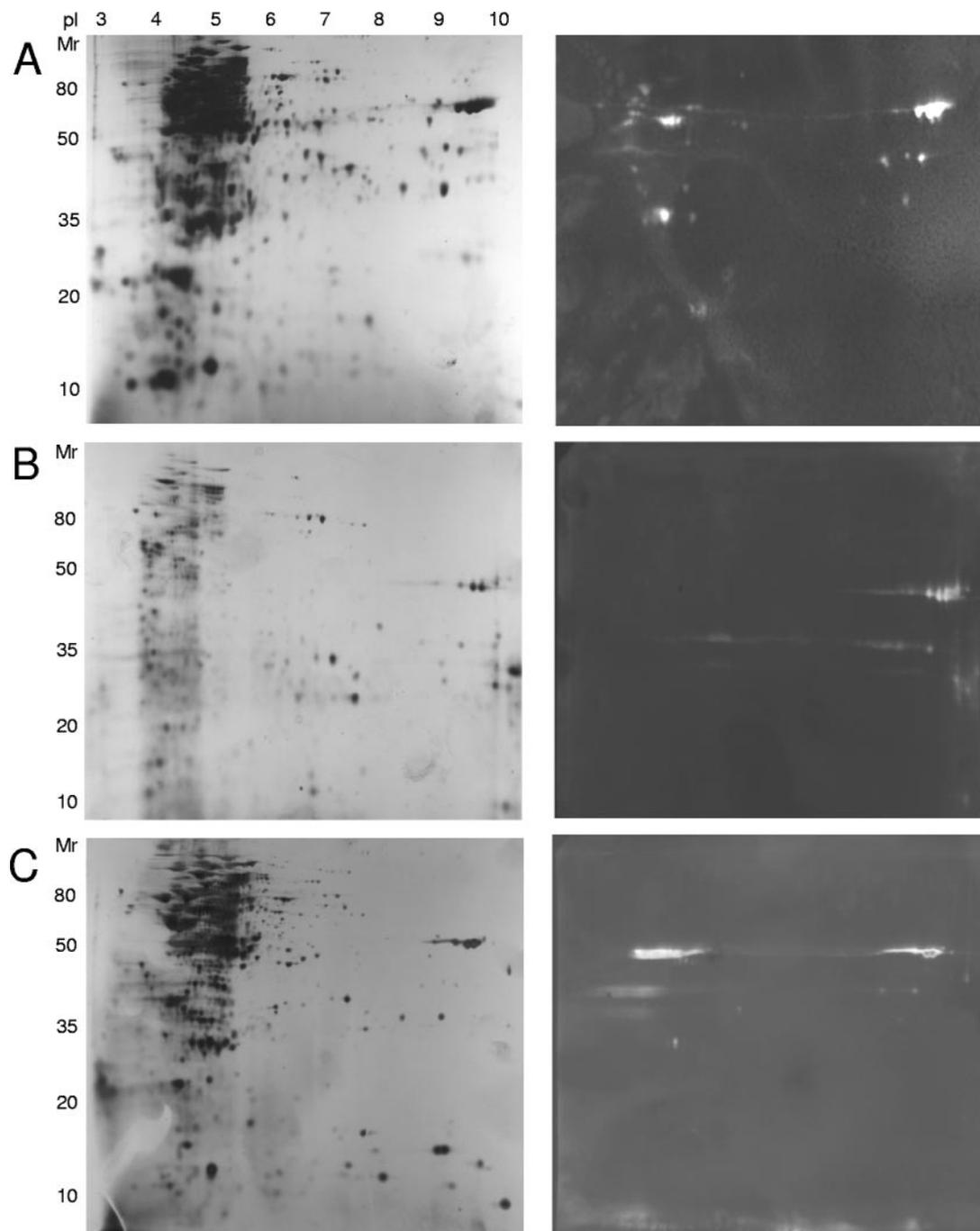
FIG. 3. Immunogenic proteins are located in all three cellular fractions of biofilm-associated *S. aureus*. Sera from a rabbit infected intratibially with *S. aureus* were utilized to visualize immunogens in the cell wall (A), membrane (B), and cytosolic (C) fractions. pIs are indicated at the top.

response to an *S. aureus* biofilm infection, we utilized 2DGE, Western blotting with sera from rabbits infected with *S. aureus*, and MALDI-TOF analysis to identify the staphylococcal immunogens present during infection. Though some work to discern the immunogens present on the surface of planktonic *S. aureus* (25, 26, 46, 53, 63, 101, 103) and the closely related *Staphylococcus epidermidis* (92) has been completed, the data presented in this paper are the first to describe biofilm-specific proteins recognized by host antibodies. Most of the immuno-

gens identified in this study are distinct from those associated with septic infection, illustrating that the antigens presented by the biofilm are different from those exhibited on the surface of planktonic *S. aureus* during acute infections.

Because of issues with contamination from host proteins during harvesting, protein expression in biofilms grown in vivo cannot be easily studied. Therefore, we employed a system by which the biofilm was cultured under in vitro conditions and a simultaneous biofilm infection was initiated in our rabbit

TABLE 2. Identities of cell wall antigens

| pI | Mol wt | Identity | Accession no. | Localization | Function |
|---|---|---|---|---|---|
| 8.7 | 44.10 | Lipase | 28195801 | Extracellular | Degradation of lipids (100) |
| 8.7 | 35.96 | Alpha-hemolysin precursor | 15924153 | Extracellular | Virulence (cell lysis) (8) |
| 9.6 | 52.56 | Autolysin | 15924043 | Extracellular | Peptidolglycan hydrolase (45) |
| 5.6 | 18.51 | Conserved hypothetical protein | 15924700 | Extracellular | Similar to that of universal stress protein family[a] |
| 5.1 | 22.69 | Superoxide dismutase | 15924543 | Extracellular | Conversion of $O_2^-$ to $H_2O_2$ and $O_2$ (41) |
| 5.2 | 26.72 | Phosphoglycerate mutase | 15925406 | Extracellular | Glycolysis (4) |
| 4.9 | 21.13 | Alkyl hydroperoxide reductase subunit C | 15923371 | Extracellular | Reduction of reactive oxygen intermediates (18) |
| 5.1 | 51.11 | Glutamine-ammonia ligase | 21282921 | Extracellular, cytosol | Ligase activity[b] |
| 5.1 | 21.55 | ATP-dependent Clp protease (ClpP) | 15923758 | Extracellular, cytosol | Sensor of stress, important in biofilm formation (30) |
| 8.7 | 34.78 | Lipoprotein | 15923621 | Extracellular | ABC transporter extracellular binding protein[c] |
| 6.6 | 76.64 | Triacylglycerol lipase precursor | 15925661 | Extracellular | Degradation of lipids (100) |
| 4.7 | 43.14 | Elongation factor Tu | 15923538 | Extracellular, cytosol | Carrier of amino acid/tRNA to the ribosome in translation (1) |
| 5.0 | 72.23 | Transketolase | 15924332 | Extracellular | Metabolism (95) |
| 4.8 | 27.41 | Triosephosphate isomerase | 15923764 | Extracellular | Glycolysis (4) |
| 5.5 | 31.74 | Hypothetical protein | 7328269 | Extracellular, cytosol | Putative thioredoxin reductase[c] |
| 5.8 | 45.33 | Serine hydroxymethyl transferase | 15925103 | Extracellular, cytosol | Synthesis of serine[b] |
| 5.6 | 33.28 | Hypothetical protein SAV1557 | 15924547 | Extracellular, cytosol | Probable endonuclease IV[a] |
| 5.7 | 37.39 | Thymidylate synthase | 27468038 | Unknown | Catalysis of 5,10-methylenetetrahydrofolate + dUMP = dihydrofolate + dTMP[b] |
| 5.4 | 26.70 | Conserved hypothetical protein | 15923245 | Extracellular | Similar to that of 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase[a] |
| 5.1 | 34.66 | Hypothetical protein | 21283953 | Cytosol | Similar to that of glycerate dehydrogenase[a] |
| 5.1 | 37.87 | Ornithine transcarbamoylase | 15925624 | Cytosol | Carbamoylation of ornithine (64) |
| 5.3 | 36.43 | Alcohol dehydrogenase I | 21282297 | Cytosol | Glycolysis (4) |

[a] Function derived from the Entrez database (www.ncbi.nih.gov).
[b] Function derived from the Expasy database (www.expasy.org).
[c] Function derived from blastP homology search (www.ncbi.nih.gov).

model with the same bacterial strain. By collecting sera from these rabbits during the course of infection and utilizing these sera to probe immunoblots of protein isolated from the in vitro-grown biofilm, we were able to visualize those proteins that were present under both conditions and were immuno-

TABLE 3. Transcriptomic profiles of cell wall-associated immunogens in biofilm versus planktonic growth

| Gene product[a] | Col strain no. | Upregulation in biofilm of indicated type[b]: | | |
|---|---|---|---|---|
| | | Early | Maturing | Mature |
| Lipase | SA0712 | \ | \ | \ |
| Alpha-hemolysin | SA1173 | + | + | − |
| Autolysin§ | SA1062 | + | + | + |
| CHP sim. to universal stress protein family | SA1759 | − | − | − |
| Superoxide dismutase§ | SA1610 | \ | − | − |
| Phosphoglycerate mutase§¶ | SA2415 | + | + | + |
| Alkyl hydroperoxide subunit C | SA0452 | − | − | − |
| Glutamine-ammonia ligase | SA1329 | − | + | + |
| Lipoprotein (ABC transporter) | SA0688 | − | + | + |
| Triacylglycerol lipase (*geh*) | SA2694 | + | + | − |
| EF-Tu | SA0594 | − | + | − |
| Transketolase | SA1377 | + | + | − |
| Hypo. thioredoxin reductase | SA0829 | \ | \ | + |
| Serine hydoxymethyl transferase | SA2105 | + | − | − |
| Hypo. endonuclease IV | SA1614 | + | \ | \ |
| Thymidylate synthase | SA1462 | + | \ | + |
| CHP | SA0240 | + | + | \ |
| Hypo. sim. to glycerate dehydrogenase | SA2296 | − | − | − |
| Alcohol dehydrogenase I¶ | SA0660 | + | + | + |
| ClpP | SA0833 | \ | \ | − |
| Ornithine transcarbamoylase§ | SA2656 | − | + | + |

[a] Symbols: § and ¶, up-regulated in biofilms (per references 5 and 4, respectively). CHP, conserved hypothetical protein; hypo., hypothetical; sim., similar.
[b] +, gene is significantly ($P \leq 0.05$) up-regulated in the biofilm versus planktonic culture; −, gene is significantly ($P \leq 0.05$) down-regulated in the biofilm versus planktonic culture; \, no change in regulation between biofilm and planktonic growth.
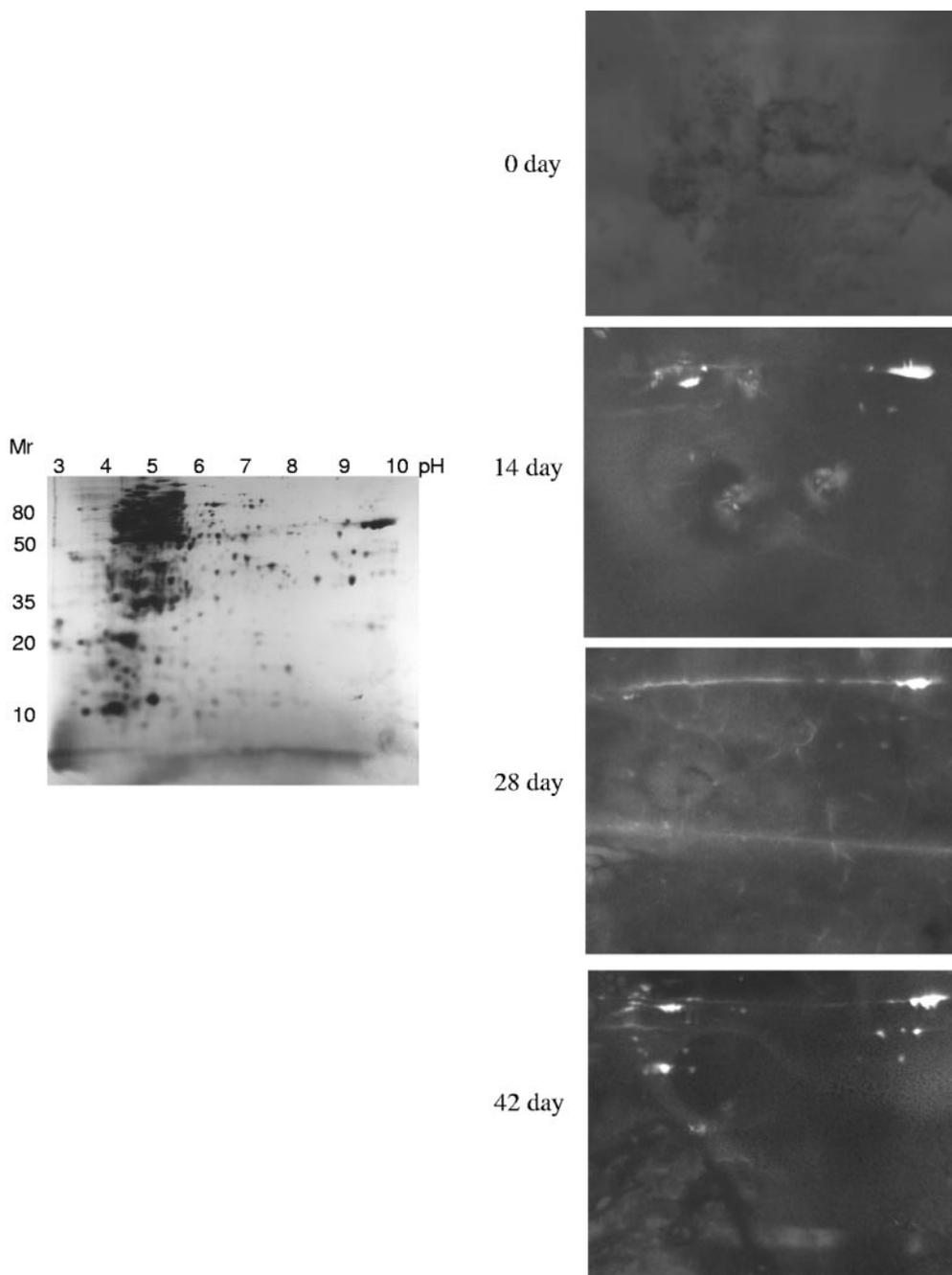
genic. It is likely that some immunogens may be down-regulated in vitro compared to the in vivo condition and thus are not present on our 2D gels. However, since the number of immunogens we identified was approximately the same as the number identified in other works (92, 101, 103), we feel that an adequate representation of the proteins present in vivo was attained. Though extremely hydrophobic proteins (such as those found in the cell membrane) can be difficult to solubilize in a manner such that they will avoid precipitation during isoelectric focusing (47, 74), the methods followed here and cited previously in the work of Nandakumar et al. were shown to be able to successfully resolve membrane proteins with up to nine transmembrane domains (65). Also, because we are most interested in those proteins that are featured on the outer cell surface and the cell membrane is buried under the gram-positive cell wall, we concentrated efforts on the cell wall protein fraction.

In our initial studies, we evaluated total protein from mechanically disrupted biofilm preparations, which normally includes a vast majority of cytosolic proteins. This was confirmed by our findings that out of 24 immunogens identified through probing mechanically disrupted *S. aureus* protein with sera from infected rabbits, 11 were strictly cytoplasmic. Therefore, this is most likely a reflection of the immune response leading to bacterial lysis, as well as simple bacterial death, thereby leading to the release of cytoplasmic contents into the host. This antigenic release possibly may act as an immune system decoy, where the immune system expends effort making antibody responses to these proteins that are not effective in the clearance of viable bacteria. These bacterial products will then be available to the immune system and indeed influence the antibodies that are created during infection. The relevance of these antibodies as active participants in eradication of such an

FIG. 4. Variation in antibody-mediated response over time. Cell wall protein (500 μg) was resolved via 2DGE, and protein was transferred to nitrocellulose membranes. The membranes were then probed with sera from days 0, 14, 28, and 42 postinoculation.

infection could be considered improbable. However, there are also several examples of proteins that were thought to be cytosolic but have been shown to be localized to the surface of bacteria, such as elongation factor Tu, alanine dehydrogenase, and serine hydroxymethyl transferase (31, 40, 61, 77, 80, 83). In addition, a recent paper by Gatlin et al. has identified 96 proteins previously believed to be intracellular as associated with the cell wall; these proteins include many of the immunogens identified in our studies (31).

In our subsequent immunogenic screen on cell wall frac-

tions, 19 of the 22 identified immunogens were found to be extracellular (either maintained in the cell wall or transiently associated therein prior to secretion). One gene product, autolysin, is involved in hydrolysis of peptidoglycan but has also been shown to be important in the attachment of cells to substrates (29, 38). The primary autolysin from *S. aureus*, Atl (28), is up-regulated under biofilm conditions compared to planktonic growth, as shown via microarray analysis. Theoretically, vaccination with autolysin could thus prevent attachment and, perhaps, biofilm formation. Lipase, a virulence fac-

TABLE 4. Proteins exhibiting varying immunogenicities over time of infection

| Protein | Accession no. | Day(s) on which immunogenicity was identified[a] |
|---|---|---|
| Lipase | 28195801 | 14, 28, 42 |
| Autolysin | 15924043 | 14, 28, 42 |
| Lipoprotein | 15923621 | 14, 28, 42 |
| Superoxide dismutase | 15924543 | 14, 28, 42 |
| Hypothetical protein | 7328269 | 14, 28, 42 |
| Transketolase | 15924332 | 42 |
| Elongation factor Tu | 15923538 | 42 |
| Triosephosphate isomerase | 15923764 | 42 |
| Alkyl hydroperoxide reductase subunit C | 15923371 | 42 |

[a] Day(s) postinoculation.

tor associated with degrading lipids (100), was seen to be highly immunogenic in the cell wall fraction, as well as in membrane and cytosolic fractions (data not shown). Other work has shown lipase to be immunogenic in human *S. aureus* infections not associated with biofilm formation (26, 103). Due to its localization and expression within biofilms, as well as its very high immunogenicity, we believe this protein is an excellent vaccine candidate. The ABC transporter lipoprotein is also a good candidate, as previous work cites it as immunogenic in *S. aureus* infections in humans (101) and as it is found on the outer portion of the bacterial cell and is up-regulated during biofilm growth.

No well-known LPXTG proteins (such as adhesins) were identified as immunogenic. However, this is not surprising, since this study was performed using mature biofilms to mimic the chronically infected in vivo condition. While cell wall-associated adhesins are commonly up-regulated in more-immature biofilms (8, 16, 48 h) compared to planktonic growths (81), these proteins are generally down-regulated in mature (7- to 14-day) biofilms (5). However, it is also possible that a number of these proteins would be difficult to identify using the methods employed in this study, as their molecular masses exceed 100 kDa. Also, because the growth conditions utilized in our in vitro biofilm system are not iron limiting, we may be missing iron acquisition proteins such as IsdA and IsdH, which were found to be immunogenic in a very recent study using sera from bacteremic patients to probe *S. aureus* proteins from an expression library (20). These LPXTG proteins were shown to prevent nasal colonization when used to vaccinate rats. Currently, ClfA is being investigated as a possible DNA vaccine candidate in mice and cattle, and while this vaccine does show some efficacy in inducing a strong humoral response and decreasing the ability of *S. aureus* to adhere to cells or fibrinogen in vitro (15, 70, 94), protection upon challenge was partial or incomplete (15, 94). Thus, LPXTG proteins are promising candidates for vaccines, but it is still worthwhile to search for other possible candidates as well.

The differential gene expression levels in planktonic versus biofilm *S. aureus* cultures have been evaluated in other studies, and it has been shown that there are many genes that are more highly expressed in the biofilm (4, 5, 81, 90). The majority of the immunogenic antigens we discovered are indeed up-regulated under biofilm conditions, as shown by microarray analysis

(Table 3). In our microarray experiment, we found that approximately 76% of the immunogenic proteins in vivo were up-regulated in at least one of the stages of biofilm formation during in vitro growth. Other researchers have evaluated the link between transcriptomic and proteomic results on identically grown *S. aureus* samples and found that genes shown to be transcriptionally up-regulated in expression were correlated with up-regulated protein production approximately 69% of the time (89). Therefore, the 76% level of agreement between the transcriptomic data derived from our in vitro-grown biofilms and the immunogens detected from an in vivo infection as detected by Western blotting of 2D gels is within ranges previously published.

Other in vitro studies have shown the up-regulation of several of our identified antigens. Becker et al. (4) determined via micro-representational-difference analysis that phosphoglycerate mutase and alcohol dehydrogenase I are up-regulated in the biofilm. Beenken et al. (5) found autolysin, phosphoglycerate mutase, alanine dehydrogenase, superoxide dismutase, and ornithine transcarbamoylase all were expressed at higher levels in biofilms than in planktonic *S. aureus*. However, the research described here enabled the identification of several additional highly expressed immunogens that previous studies failed to detect. We were also able to simultaneously link the proteomic and transcriptomic results from an in vitro biofilm growth model with more-applicable information about what is present in vivo for the first time.

Previous in vivo studies that evaluated the immunogenicity of *S. aureus* proteins in acute infections that represent planktonic growth conditions (such as sepsis) have also been performed (26, 53, 63, 101, 103). However, these studies failed to detect the biofilm-associated antigens found in this work, with the exception of autolysin and lipase. This underlines the importance of determining antigens that either are present at high levels under both conditions or are at high levels under biofilm conditions for the study of biofilm-associated infections.

By probing proteins from the cell wall fraction of a mature *S. aureus* biofilm with sera from uninfected rabbits as well as with sera from rabbits at various time points after infection, we were able to monitor the changes in antibody response to the *S. aureus* biofilm osteomyelitis infection as it moved from an acute to a chronic state. Over the course of infection, immunogenic spots increased in number on the Western blots, indicating that additional B- and T-cell antigens are up-regulated as the biofilm matures. We saw the majority of immunogenic spots were present at day 42 postinoculation. Some of the most immunogenic proteins, lipase, autolysin, and superoxide dismutase, were present at every time postinoculation. However, several others were immunogenic only at day 42 postinoculation. As the biofilm is maturing and entering a chronic state of disease, the proteins that are being recognized by the immune system are changing. If the biofilm were simply mimicking an acute infection, we would not see these changes and instead would see many more antigens that are reflected in the aforementioned works in which acute *S. aureus* infections were studied. While work looking at the transcriptional profile of *S. aureus* under biofilm conditions at certain time points besides that presented here has been completed (81), thus far no work to determine what proteins are expressed by *S. aureus* biofilms

in vivo and, in particular, at what time points during infection these proteins are made and/or exposed on the bacterial surface has yet been completed.

The temporal immunogenicity of the antigens discovered in this work could give further insight into when these proteins are exposed on the surface of *S. aureus* while within a biofilm in vivo. This could be useful in designing novel therapeutics for biofilm infections. Those antigens that are recognized early during the immune response (such as lipase or autolysin) could be considered as promising vaccine targets, while the late antigens (e.g., transketolase) may be efficacious for adjuvant therapy. One may question whether a vaccine is able to prevent a biofilm infection, especially considering that this study has shown that a significant antibody response occurs but is still ineffective. However, it may be that priming the immune system to recognize early biofilm antigens and to mount a significant response against the infection before it progresses to the mature, chronic biofilm form may prove to be successful. In fact, a number of studies have shown previously that an antibody-mediated response is effective at clearing a biofilm infection in the early phase of formation (66, 94, 97). However, this antibody-mediated response is shut down both by the host cytokines associated with the initial response to *S. aureus*, most notably gamma interferon (3, 48, 86), and by *S. aureus*' production of superantigens, capsule, and other toxins (2, 9, 35, 68, 76, 78, 98). By the time that the antibody-mediated immune system recovers and mounts a response against the biofilm infection, the fully mature biofilm is able to resist clearance. Therefore, biofilm-up-regulated antigens, when given as a vaccine, may enable the host adaptive immune system to shift to the more effective antibody-mediated response. This enables the host to escape the development of a biofilm-mediated chronic infection. Adjuvant therapy would be useful in cases of established osteomyelitis or implant infection in order to stimulate a biofilm-specific immune response that could perhaps reduce persistence by the mature biofilm.

Capsules are an important immunoavoidance mechanism of planktonic S. *aureus* (72, 82), and polysaccharide intercellular adhesin/poly-*N*-acetylglucosamine (PNAG) is the primary carbohydrate component of the biofilm (22, 27, 59). Because we utilized 2DGE and looked only at the immunogenicity of proteins in our analysis, we may have missed these carbohydrate components as antigens. Clinical trials of a capsule conjugate vaccine (StaphVax) for prevention of staphylococcal sepsis are under way. However, the efficacy of this vaccine in preventing bacteremia is only approximately 60% and lasted only through the early weeks of the study (93). Vaccines against polysaccharide intercellular adhesin/PNAG are currently in the developmental stages. Early work showed extremely variable immunoglobulin G responses in both mice and rabbits. A recent study showed that conjugating PNAG to diphtheria toxin and deacetylating it led to a more robust and protective response (60). However, *ica* expression has been shown to be dispensable in biofilm formation, so a PNAG vaccine may not be effective against some isolates. Therefore, while two main carbohydrates of *S. aureus* are already being pursued for use as vaccines, the search for possible alternative candidate proteins for use in a vaccine is of great importance.

Those antigens that are present in the cell wall fraction are promising vaccine candidates. Because we have seen that an immune response is indeed generated against these proteins, there is no reason to suspect that the same response could not be generated against purified recombinant forms. Work is currently in process in our laboratory to express recombinant forms of several of these proteins and test their efficacy in preventing osteomyelitis infection in vaccinated rabbits.

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