Coagulases as Determinants of Protective Immune Responses against *Staphylococcus aureus*

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During infection, *Staphylococcus aureus* secretes two coagulases (Coa and von Willebrand factor binding protein [vWbp]), which, following an association with host prothrombin and fibrinogen, form fibrin clots and enable the establishment of staphylococcal disease. Within the genomes of different *S. aureus* isolates, coagulase gene sequences are variable, and this has been exploited for a classification of types. We show here that antibodies directed against the variable prothrombin binding portion of coagulases confer type-specific immunity through the neutralization of *S. aureus* clotting activity and protection from staphylococcal disease in mice. By combining variable portions of coagulases from North American isolates into hybrid Coa and vWbp proteins, a subunit vaccine that provided protection against challenge with different coagulase-type *S. aureus* strains in mice was derived.

*S*taphylococcus aureus, a Gram-positive microbe that colonizes the human skin and nares, also causes invasive diseases such as skin and soft tissue infections, bacteremia, sepsis, and endocarditis (27). The emergence of methicillin-resistant strains in the community (community-acquired methicillin-resistant *S. aureus* [CA-MRSA]) and the increase in rates of hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) present a formidable therapeutic challenge (18). Although several vaccine development efforts have been launched, an FDA-licensed *S. aureus* vaccine is not yet available (8).

A hallmark of *S. aureus* isolates is their ability to form clots when inoculated into human citrate-plasma or blood (31). This phenotype has been linked to the secretion of coagulase (Coa) (6), which binds prothrombin and alters the enzyme's active site through the insertion of its N-terminal residues into the activation pocket, thereby providing for the cleavage of fibrinogen to fibrin (12). The mature form of Coa is comprised of N-terminal D1 and D2 domains, which enable the association with and activation of prothrombin (33) (Fig. 1). A linker (L) domain connects D12 and the repeat (R) region, comprised of tandem repeats of a 27-residue peptide that bind fibrinogen (34) (Fig. 1). The prothrombin·Coa complex (staphylocoagulase) converts soluble fibrinogen to insoluble fibrin, forming the mesh network of a clot (12, 21).

When injected into animals, purified Coa clots blood *in vivo*, and this is thought to promote staphylococcal escape from phagocytic killing (13, 16). Coagulase typing, i.e., the neutralization of *S. aureus* coagulation of citrate-plasma with specific antiserum, has been used to distinguish 10 different serological Coa types (17). Coa types have also been analyzed by DNA sequencing, which revealed significant variation within *coa* sequences for the D12 domain and little variation for the linker and repeat regions, respectively (44). Is sequence variation within *S. aureus coa* genes the result of negative selection, as might occur when infected individuals develop antibody responses against secreted Coa? To begin to address this question, Watanabe and colleagues sequenced the *coa* genes from 126 *S. aureus* isolates, simultaneously analyzing them for coagulase serotype and clonal cluster (CC) type (44). The latter is accomplished via multilocus sequence typing (MLST), which

examines sequences from seven different genes (*arc*, *aro*, *glp*, *gmk*, *pta*, *tpi*, and *yqi*) (11). With the exception of CC1 and CC8 strains, which frequently harbor different *coa* types, most of the isolates categorized as a single CC type by MLST also harbor a single *coa* sequence type (43). The variation of *coa* sequences is likely generated via horizontal gene transfer (for example, phage transduction or DNA transformation), because *coa* genes of the same sequence type are found scattered across the MLST tree (43). Together with the observation that pooled human immunoglobulin neutralizes most, but not all, coagulase types (40), these analyses suggest that *coa* gene diversification may enable *S. aureus* to circumvent the humoral immune responses of hosts with prior exposure to the pathogen (43). If so, Coa may represent a protective antigen of *S. aureus* and should be analyzed for its possible use as a vaccine antigen.

Nearly a century after the first description of staphylococcal coagulase, Bjerketorp and colleagues discovered von Willebrand factor (vWF) binding protein (vWbp) (3). vWbp is a secreted protein that, in addition to binding vWF, also associates with prothrombin to convert fibrinogen to fibrin (2, 12, 21). vWbp displays sequence homology to the Coa D12 domains (2, 44). However, its C-terminal domain lacks the L and R domains of Coa, which are replaced by unique vWF and fibrinogen binding sites (3, 6). Genome sequencing of prominent clinical strains discovered two distinct *vwb* alleles with variation in the predicted D12 domains (44). The immunization of mice with purified recombinant Coa and vWbp provides an additive level of protection against
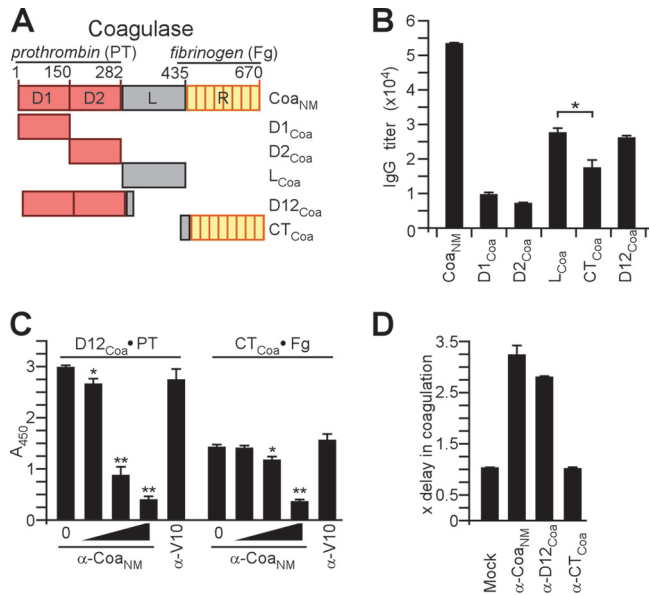
FIG 1 Immune responses to coagulase. (A) Drawing to illustrate the primary structure of coagulase from *S. aureus* Newman (Coa$_{NM}$), which was purified from *E. coli* via an N-terminal His$_6$ tag. Coa$_{NM}$ encompasses the D1 and D2 domains involved in prothrombin (PT) binding, the linker (L) domain, and the repeat (R) domain, which is comprised of tandem repeats of a 27-residue peptide sequence that binds to fibrinogen (Fg). In addition to Coa$_{NM}$, the D1$_{Coa}$, D2$_{Coa}$, D12$_{Coa}$, L$_{Coa}$, and R$_{Coa}$ domains were purified. (B) Rabbits were immunized with purified Coa$_{NM}$, and immune sera were examined by an ELISA for serum IgG reactive with Coa$_{NM}$, D1$_{Coa}$, D2$_{Coa}$, D12$_{Coa}$, L$_{Coa}$, or CT$_{Coa}$. Statistical analysis was performed with the Student two-tailed *t* test (*, $P < 0.05$). (C) The association of D12$_{Coa}$ with human prothrombin was measured by an ELISA and perturbed with increasing concentrations of rabbit IgG directed against Coa$_{NM}$ (0 µM, 0.012 µM, 0.12 µM, or 1.2 µM) or the plague vaccine antigen V10 (1.2 µM) as a control. The association of CT$_{Coa}$ with fibrinogen was measured by an ELISA and perturbed with increasing concentrations of rabbit IgG directed against Coa$_{NM}$ (0 µM, 0.017 µM, 0.17 µM, or 1.7 µM) or the plague vaccine antigen V10 (1.7 µM) as a control. (D) Affinity-purified rabbit IgG specific for Coa$_{NM}$ (α-Coa$_{NM}$), D12$_{Coa}$ (α-D12$_{Coa}$), or CT$_{Coa}$ (α-CT$_{Coa}$) was added to citrate-treated mouse blood and inoculated with *S. aureus* Newman to monitor the inhibition of staphylococcal coagulation.

challenge with the same coagulase-type *S. aureus* strain in animal models of abscess formation and lethal bacteremia (6). *S. aureus* Newman mutants lacking *coa* and *vwb*, but not variants with single-gene deletions, displayed significant defects in mouse models of abscess formation or lethal bacteremia (6). The secretion of Coa and vWbp enables *S. aureus* to agglutinate in the presence of plasma, resulting in thromboembolic lesions as well as endocarditis and promoting the lethal outcome of staphylococcal bacteremia (28, 35). The blocking of coagulases with univalent direct thrombin inhibitors delays the time to death associated with lethal *S. aureus* challenge, further highlighting the importance of coagulases in staphylococcal disease (28).

Thus, Coa and vWbp promote the pathogenesis of *S. aureus* abscess formation and lethal bacteremia in mice by promoting staphylococcal coagulation and agglutination. If so, the antibody-mediated neutralization of Coa and vWbp may provide protection from staphylococcal disease, which could be exploited for the development of vaccines. Here we examined immune responses to coagulases and report that antibodies against the D12 domain neutralize staphylococcal coagulation in a type-specific manner. By injecting mice with a vaccine composed of four Coa-type D12 domains and two vWbp-type D12 domains (Coa$_4$/vWbp$_2$) that harbors antigenic determinants from the major North American isolates (CC1 [USA400], CC5 [USA100], CC8 [USA300], CC30, and CC45) (19, 36), mice could be protected against challenge with several different *S. aureus* strains.

MATERIALS AND METHODS

Ethics statement. Experiments with blood from human volunteers involved a protocol that was reviewed, approved, and performed under the regulatory supervision of the University of Chicago's Institutional Review Board (IRB). Written, informed consent was provided by all volunteers. Animal experiments involving *S. aureus* challenge followed protocols that were reviewed, approved, and performed under the regulatory supervision of the University of Chicago's Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC). Animals were managed by the University of Chicago Animal Resource Center, which is accredited by the American Association for Accreditation of Laboratory Animal Care and the Department of Health and Human Services (DHHS) (protocol A3523-01). Animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the DHHS *Guide for the Care and Use of Laboratory Animals* (31a). Veterinary Care was under the direction of full-time resident veterinarians boarded by the American College of Laboratory Animal Medicine. BALB/c mice and New Zealand White rabbits were purchased from Charles River Laboratories and Harlan Sprague-Dawley, respectively. The statistical analysis of staphylococcal sepsis was performed by using the two-tailed log rank test. The results of all animal experiments were examined for reproducibility.

Bacterial strains and growth of cultures. *S. aureus* strains were cultured on tryptic soy agar or broth at 37°C. *Escherichia coli* strains DH5α and BL21(DE3) were cultured on Luria-Bertani agar or broth at 37°C. Ampicillin (100 µg/ml) was used for pET15b and pGEX2tk selection. Primers used for the amplification of staphylococcal DNA are found in Table S1 in the supplemental material.

Coa$_4$ and vWbp$_2$. To generate the hybrid proteins, *coa* and *vwb* from strain USA300 were PCR amplified. The 5′ primer included the restriction site (NcoI) to insert onto the vector (pET15b) as well as an additional restriction enzyme (AvrII) for future use. The 3′ primer included the restriction site (BamHI) for vector insertion. The inserts were cloned into *E. coli* strain DH5α. In each subsequent round of cloning, the D12 region from the next allele was added 5′ to the previous insert. In each case, the 5′ primer included the vector site (NcoI) and an additional restriction enzyme site for future use. The 3′ primer for each sequential insert contained the restriction site (AvrII for N315) included in the 5′ primer for the previous insert. The promoter region and His tag were restored in a subsequent round of cloning, and a C-terminal Strep tag was added in another round of cloning. The entire vector was sequenced to verify the DNA sequence. Finally, each vector was transformed into *E. coli* strain BL21 for protein expression and purification.

Protein purification. *E. coli* BL21(DE3) cells harboring expression vectors (containing *coa* from *S. aureus* Newman; *vwb* from *S. aureus* strains Newman, USA300 and N315; or subdomains of *coa* and *vwb* as well as expression vectors containing the genetic sequence for the hybrid proteins Coa$_4$ and vWbp$_2$) were grown at 37°C and induced with 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at room temperature. Because of degradation during the purification of Coa, pGEX2tk expression vectors in *E. coli* DH5α were used to express *coa* from USA300, N315, MW2, MRSA252, 85/2082, and WIS as glutathione *S*-transferase (GST)-tagged constructs. At 3 h following induction, cells were centrifuged at 7,000 × *g*, suspended in 1× column buffer (0.1 M Tris-HCl [pH 7.5], 0.5 M NaCl), and lysed in a French pressure cell at 14,000 lb/in². Lysates were subjected to ultracentrifugation at 40,000 × *g* for 30 min. The supernatant of pET15b constructs was subjected to Ni-nitrilotriacetic acid (NTA) chromatography, washed with column buffer and 10 mM imidazole, and eluted with 500 mM imidazole. For Strep-tagged proteins, lysate

TABLE 1 Type-specific or cross-protective inhibition of staphylococcal coagulation by coagulase antibodies[a]

Coa type	CC type	Strain	Mean fold delay in time to clotting with antibody (SEM)				
			Mock	Anti-Newman	Anti-N315	Anti-MW2	Anti-MRSA252
IIa	5	N315	1.0 (0.0)	1.8 (0.7)	1.6 (0.4)	1.7 (0.5)	1.3 (0.2)
IIa	5	Mu50	1.0 (0.0)	1.0 (0.0)	1.2 (0.2)	1.2 (0.2)	1.1 (0.3)
IIIa	8	Newman	1.0 (0.0)	2.2 (0.5)	1.6 (0.4)	2.3 (0.6)	1.2 (0.1)
IIIa	8	USA300	1.0 (0.0)	1.5 (0.6)	1.3 (0.3)	1.6 (0.7)	1.4 (0.4)
IVa	30	MRSA252	1.0 (0.0)	0.9 (0.1)	1.3 (0.0)	1.1 (0.1)	0.9 (0.1)
IVa	30	CowanI	1.0 (0.0)	0.9 (0.1)	1.0 (0.0)	1.0 (0.1)	0.8 (0.3)
VIIa	1	MW2	1.0 (0.0)	1.2 (0.3)	0.9 (0.1)	1.5 (0.3)	1.1 (0.1)
VIIa	1	MSSA476	1.0 (0.0)	1.0 (0.1)	0.9 (0.1)	1.3 (0.1)	1.1 (0.4)

[a] Calcium-chelated mouse blood was inoculated with 1×10^6 CFU of the indicated strain of *S. aureus* mixed with PBS or anti-Coa antibody from strain Newman, MW2, N315, or MRSA252 (3 μM final concentration); incubated at 37°C; and monitored over time. Values represent fold delays in the time to clotting, calculated as the length of time for the clot to form in the presence of the antibody divided by the absence of antibody for that strain. Values in parentheses reflect the standard errors of the mean from three or four independent experiments. A 1.5-fold or greater delay in staphylococcal coagulation was considered a significant reduction in the clotting time.

supernatants were subjected to chromatography over StrepTactin-Sepharose (GE), washed in 1× Strep wash buffer (0.1 M Tris-HCl [pH 8.0], 0.150 M NaCl, 0.1 M EDTA), and eluted in 1× Strep wash buffer containing 2.5 mM desthiobiotin. For GST-tagged proteins, the supernatant of cleared lysates was subjected to glutathione-Sepharose chromatography. To remove the GST tag, following washing with column buffer, the column buffer was switched to PreScission protease cleavage buffer containing 10 mM dithiothreitol (DTT), and the column was incubated with PreScission protease (GE Healthcare) overnight at the unit definition provided by GE. Liberated protein lacking the GST tag was then collected with additional protease cleavage buffer. Eluates were dialyzed against phosphate-buffered saline (PBS). To remove endotoxin, a 1:100 dilution of Triton X-114 was added, and the solution was chilled for 10 min, incubated at 37°C for 10 min, and centrifuged at $13,000 \times g$. This was repeated twice. The supernatant was loaded onto a HiTrap desalting column to remove remnants of Triton X-114.

Rabbit antibodies. The protein concentration was determined by using a bicinchoninic acid (BCA) kit (Pierce). Purity was verified by SDS-PAGE analysis and Coomassie brilliant blue staining. Six-month-old New Zealand White female rabbits were immunized with 500 μg protein emulsified in complete Freund's adjuvant (CFA) (Difco) for initial immunization or incomplete Freund's adjuvant (IFA) for booster immunizations on days 24 and 48. On day 60, rabbits were bled, and serum was recovered for immunoblotting or passive-transfer experiments. For antibody purification, recombinant His$_6$-Coa, His$_6$-vWbp, or His$_6$-ClfA (5 mg) was covalently linked to HiTrap *N*-hydroxysuccinimide (NHS)-activated high-performance (HP) columns (GE Healthcare). This antigen matrix was then used for affinity chromatography of 10 to 20 ml of rabbit serum at 4°C. The charged matrix was washed with 50 column volumes of PBS, and antibodies were eluted with elution buffer (1 M glycine [pH 2.5], 0.5 M NaCl) and immediately neutralized with 1 M Tris-HCl (pH 8.5). Purified antibodies were dialyzed overnight against PBS–0.5 M NaCl at 4°C.

Coagulation assay. Cultures of staphylococcal strains grown overnight were diluted 1:100 into fresh tryptic soy broth (TSB) and grown at 37°C until they reached an optical density at 600 nm (OD$_{600}$) of 0.4. One milliliter of culture was centrifuged, and staphylococci were washed and suspended in 1 ml of sterile PBS to generate a suspension of 1×10^8 CFU/ml. Whole blood from naïve BALB/c mice was collected, and sodium citrate was added to a final concentration of 1% (wt/vol). To assess bacterial blood-coagulating activity in the presence of antibodies, 10 μl of the stock bacterial culture was mixed with 10 μl of PBS containing a 30 μM anti-Coa and anti-vWbp mixture in a sterile plastic test tube (BD Falcon) and incubated for 15 min. To each tube, 80 μl of anti-coagulated mouse blood was added in a sterile plastic test tube (BD falcon) to achieve a final concentration of 1×10^7 CFU/ml. Test tubes were incubated at 37°C, and blood coagulation was verified by tipping the tubes to 45° angles at timed intervals. All experiments were performed twice for reproducibility.

Active immunization. Three-week-old BALB/c mice ($n = 10$) were injected with 50 μg protein emulsified in incomplete Freund's adjuvant and complete Freund's adjuvant (3:2). At 11 days postvaccination, these mice were boosted with 50 μg protein, each emulsified in incomplete Freund's adjuvant. On day 21, mice were anesthetized with ketamine-xylazine, and blood was collected by retro-orbital bleeding using microhematocrit capillary tubes (Fisher) in Z-Gel microtubes (Sarstedt) for determining half-maximal titers. Tubes were centrifuged at $10,000 \times g$ for 3 min, and serum was collected. Half-maximal antibody titers were measured by an enzyme-linked immunosorbent assay (ELISA).

Passive transfer of antibodies. Six hours prior to infection, 6-week-old BALB/c mice ($n = 10$) were injected intraperitoneally with affinity-purified antibodies against full-length or subdomain constructs of Coa or vWbp or against V10 (control IgG specific for the LcrV plague antigen) at a dose of 5 mg/kg of body weight.

Sepsis. Cultures of staphylococcal strains grown overnight were diluted 1:100 into fresh TSB and grown until they reached an OD$_{600}$ of 0.4. Bacteria were centrifuged at $7,000 \times g$, washed, and suspended in a 1/10 volume of PBS. Six-week-old female BALB/c mice ($n = 10$) (Charles River) were injected retro-orbitally with suspensions containing 1×10^8 CFU (*S. aureus* Newman, N315, and WIS), 5×10^7 CFU (*S. aureus* USA300), or 2×10^8 CFU (*S. aureus* MW2 and CowanI) in 100 μl of PBS. Mice were monitored for survival over 10 days.

Renal abscess. *S. aureus* strains were prepared as described above for sepsis, but following washing, bacterial pellets were suspended in an equal volume, resulting in 1-log-fewer CFU than for sepsis. To enumerate the staphylococcal load in kidney tissue at 5 days postinfection, mice were euthanized by CO$_2$ asphyxiation, and kidneys were removed during necropsy. One kidney per mouse was homogenized in PBS–1% Triton X-100. Serial dilutions of homogenate were spread onto tryptic soy agar (TSA) and incubated for colony formation. The bacterial load in tissue was analyzed by pairwise comparisons between wild-type and mutant strains with the unpaired two-tailed Student *t* test. For histopathology, the alternate kidney was fixed in 10% formalin for 24 h at room temperature. Tissues were embedded in paraffin, thin sectioned, stained with hematoxylin and eosin, and examined by light microscopy to enumerate pathological lesions per organ. Data were analyzed in pairwise comparisons between wild-type and mutant strains with the unpaired two-tailed Student *t* test.

Coagulase activity. Purified recombinant Coa or vWbp (100 nM) was mixed with human prothrombin (Innovative Research) in 1% sodium citrate–PBS. After an initial reading, fibrinogen (3 μM) (Sigma) was added, and the conversion of fibrinogen to fibrin was measured as the increase in the turbidity at 450 nm in a plate reader (BioTek) at 2.5-min intervals. As controls, the enzymatic activity of human alpha-thrombin (Innovative Research) or prothrombin alone was measured.

RESULTS

Antibodies against coagulase domains. Rabbits were immunized with affinity-purified His-tagged Coa derived from the coagulase gene of *S. aureus* Newman (Coa_{NM}). Coa_{NM}-specific immune serum was examined by an ELISA, which revealed serum IgG antibody responses to antigen (Fig. 1A and B). To analyze the antibody responses against specific subdomains, affinity-purified recombinant proteins ($D1_{Coa}$, $D2_{Coa}$, $D12_{Coa}$, L_{Coa}, and CT_{Coa}) were subjected to an ELISA (Fig. 1B). Immune serum harbored antibodies against each of the domains tested (Fig. 1B). Of note, antibodies against L_{Coa} were more abundant than antibodies that recognized the repeat domain (CT_{Coa}) ($P < 0.05$ for L_{Coa} versus CT_{Coa}). Antibodies against $D12_{Coa}$ were more abundant than those that recognized the repeat domain, but this difference did not achieve statistical significance. To probe the biological function of antibodies in rabbit immune serum, we used variable amounts of affinity-purified Coa_{NM} antibodies to perturb the association of $D12_{Coa}$ with human prothrombin or the association of CT_{Coa} with fibrinogen (Fig. 1C). We calculated that 120 nM anti-Coa IgG blocked $D12_{Coa}$ binding to prothrombin, whereas 1.7 μM anti-Coa IgG blocked the association of CT_{Coa} with fibrinogen (Fig. 1C).

Rabbit Coa_{NM} immune serum was subjected to affinity chromatography using either full-length Coa_{NM} (anti-Coa_{NM} antibody), $D12_{Coa}$ (anti-$D12_{Coa}$ antibody), or CT_{Coa} (anti-CT_{Coa} antibody). Equimolar amounts of affinity-purified IgG were added to citrate-blood samples obtained from naïve BALB/c mice, which were subsequently inoculated with *S. aureus* CC8 strain Newman (1). Compared to control samples without antibody, the addition of either anti-Coa_{NM} or anti-$D12_{Coa}$ IgG caused a significant delay in the clotting time, whereas the anti-CT_{Coa} antibody did not (Fig. 1D). Thus, rabbits respond to immunization with Coa_{NM} by generating antigen-specific IgG molecules that are directed predominantly against $D12_{Coa}$ and L_{Coa} and interfere with the clotting activity of secreted Coa. In contrast, antibodies against CT_{Coa} are generated in a lesser abundance and do not interfere with *S. aureus* Newman *in vitro* blood coagulation.

Type-specific and cross-protective inhibition of *S. aureus* coagulation. To examine the ability of the anti-Coa_{NM} antibody to block the coagulation of other strains isolated from human infections, antigen-specific IgG was added to citrate-blood samples from naïve mice that were subsequently inoculated with *S. aureus* N315 (CC5), Mu50 (CC5), Newman (CC8), USA300 (CC8), MRSA252 (CC30), CowanI (CC30), MW2 (CC1), or MSSA476 (CC1) (Table 1). Coa_{NM}-specific IgG delayed clotting by *S. aureus* Newman (CC8), USA300 (CC8), and N315 (CC5) but not clotting by MW2 (CC1), MSSA476 (CC1), Mu50 (CC5), MRSA252 (CC30), or CowanI (CC3) (Table 1). A 1.5-fold or greater delay in staphylococcal coagulation was considered a significant reduction in the clotting time. These results suggested that antibodies against Coa_{NM} not only interfere with the coagulation of *S. aureus* strains from the same CC type (or Coa type) but also may interfere with the coagulation of strains of other types (N315). To examine the generality of type-specific and cross-protective inhibition, Coa_{N315}, Coa_{MW2}, and $Coa_{MRSA252}$ were purified, and rabbit immune sera were generated (Table 1). Coa_{N315}-specific IgG inhibited the coagulation of *S. aureus* N315 (CC5) and Newman (CC8). Antibodies directed against Coa_{MW2} inhibited the clotting of *S. aureus* MW2 (CC1), N315 (CC5), Newman (CC8), and USA300
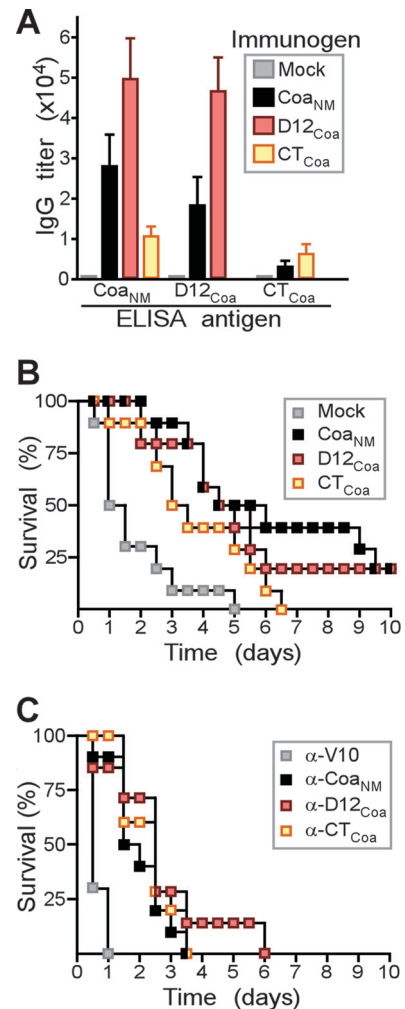
FIG 2 Coagulase domains as vaccine antigens. (A) Recombinant purified Coa_{NM}, $D12_{Coa}$, and CT_{Coa} were used to immunize BALB/c mice ($n = 5$) with a prime-boost regimen, and immune sera were analyzed by an ELISA for the reactivity of mouse serum IgG toward purified Coa_{NM}, $D12_{Coa}$, or CT_{Coa}. (B) Cohorts of BALB/c mice ($n = 10$) were immunized with a prime-boost regimen of purified Coa_{NM}, $D12_{Coa}$, and CT_{Coa} and challenged by intravenous injection with *S. aureus* Newman (1×10^8 CFU). The survival of animals was monitored over 10 days. (C) Affinity-purified rabbit IgG specific for Coa_{NM} (α-Coa_{NM}), $D12_{Coa}$ (α-$D12_{Coa}$), CT_{Coa} (α-CT_{Coa}), or V10 (α-V10) was injected at a concentration of 5 mg/kg of body weight into the peritoneal cavity of naïve BALB/c mice. Passively immunized mice were challenged by intravenous injection with *S. aureus* Newman (1×10^8 CFU), and the survival of animals was monitored over 10 days.

(CC8). Antibodies against $Coa_{MRSA252}$ had very little activity in this assay. The coagulation of mouse blood by *S. aureus* strains was inhibited by antibodies raised against the corresponding Coa, with the exception of the CC30 antibody (CC8, CC5, and CC1 isolates). Cross-neutralization of coagulation was observed for antibodies directed against the coagulase from the CC8 strain and for the coagulases of CC1 and CC5 strains. Finally, antibodies directed against Coa from the CC1, CC5, CC8, CC30, and CC45 strains did not neutralize the clotting of *S. aureus* strain Mu50 (CC5), CowanI (CC30), or MSSA476 (CC30). We presume that blood clotting in these isolates may depend predominantly on another factor, for example, vWbp (see below).

Coagulase antibodies and their protective effect against staphylococcal disease. Purified Coa_{NM}, $D12_{Coa}$, or CT_{Coa} was emulsified and injected as a prime-boost regimen into BALB/c mice ($n = 10$). Sera of mock (PBS)-, Coa_{NM}-, $D12_{Coa}$-, or CT_{Coa}-immunized animals were examined by an ELISA for IgG responses to antigen, which were detected in vaccinated animals but not in control mice (Fig. 2A and B). The immunization of mice with Coa_{NM} raised antibodies directed predominantly against $D12_{Coa}$ and, to a lesser degree, against CT_{Coa} (Fig. 2A). $D12_{Coa}$ immunization raised high-titer antibodies that reacted with full-length Coa_{NM} (Fig. 2A). In contrast, CT_{Coa} immunization generated weak antibody responses (Fig. 2A). Mice were challenged by intravenous injection with *S. aureus* Newman and observed for 10 days to assess protection against lethal sepsis (Fig. 2B). Compared to mock-immunized animals, vaccination with Coa_{NM}, $D12_{Coa}$, or CT_{Coa} resulted in an increased time to death ($P < 0.001$ for Coa_{NM} versus PBS, $P < 0.01$ for $D12_{Coa}$ versus PBS, and $P < 0.05$ for CT_{Coa} versus PBS). Immune responses against Coa_{NM} did not generate increased protection compared to either $D12_{Coa}$ or CT_{Coa} vaccination ($P > 0.05$ for Coa_{NM} versus CT_{Coa} and $P > 0.05$ for $D12_{Coa}$ versus CT_{Coa}).

We asked whether antibodies directed against $D12_{Coa}$ or CT_{Coa} provide protection against a lethal *S. aureus* challenge. Affinity-purified rabbit IgG was injected into the peritoneal cavity of naïve BALB/c mice at a concentration of 5 mg/kg of body weight (Fig. 2C). Four hours later, animals were challenged by the intravenous injection of *S. aureus* Newman (Fig. 2C). Compared to control antibodies specific for the V10 plague protective antigen (7) (anti-V10 antibody), IgGs directed against Coa_{NM}, $D12_{Coa}$, or CT_{Coa} each caused a delay in the time to death for the corresponding cohort of challenged animals ($P < 0.05$ for all vaccines versus PBS) (Fig. 2C). No significant differences in disease protection were detected among antibodies directed against $D12_{Coa}$, CT_{Coa}, and full-length Coa_{NM} (Fig. 2C). Thus, compared to $D12_{Coa}$ immunization, the CT_{Coa} domain elicits low antibody responses. However, the passive transfer of antibodies against $D12_{Coa}$ and CT_{Coa} provided similar levels of protection against a lethal *S. aureus* Newman challenge. These data suggest that the antibody-mediated neutralization of *S. aureus* Newman coagulase activity may not be an absolute prerequisite for disease protection. Following exposure to full-length Coa_{NM}, BALB/c mice mount robust immune responses against $D12_{Coa}$ and L_{Coa} but generate few antibodies against CT_{Coa}.

Antibodies against von Willebrand factor binding protein domains. Rabbits were immunized with affinity-purified His-tagged vWbp derived from the *vwb* gene of *S. aureus* Newman ($vWbp_{NM}$). Immune serum was examined by an ELISA, which revealed serum IgG antibody responses to antigen (Fig. 3A and B). To analyze antibody responses against specific subdomains, affinity-purified $D1_{vWbp}$, $D2_{vWbp}$, $D12_{vWbp}$, CT_{1vWbp}, and CT_{vWbp} were subjected to an ELISA (Fig. 3B). Immune serum harbored antibodies against each subdomain tested (Fig. 3B). Of note, antibodies against $D1_{vWbp}$ and $D2_{vWbp}$ were less abundant than antibodies that recognized both domains together ($D12_{vWbp}$). Compared to immune responses against $D12_{vWbp}$, antibodies directed against CT_{vWbp} were 30% less abundant ($P > 0.05$ for $D12_{vWbp}$ versus CT_{vWbp}). To probe the biological function of antibodies in the immune serum, we used variable amounts of $vWbp_{NM}$-specific IgG to perturb the association of $D12_{vWbp}$ with human prothrombin and the association of CT_{vWbp} with fibrinogen (Fig. 3C
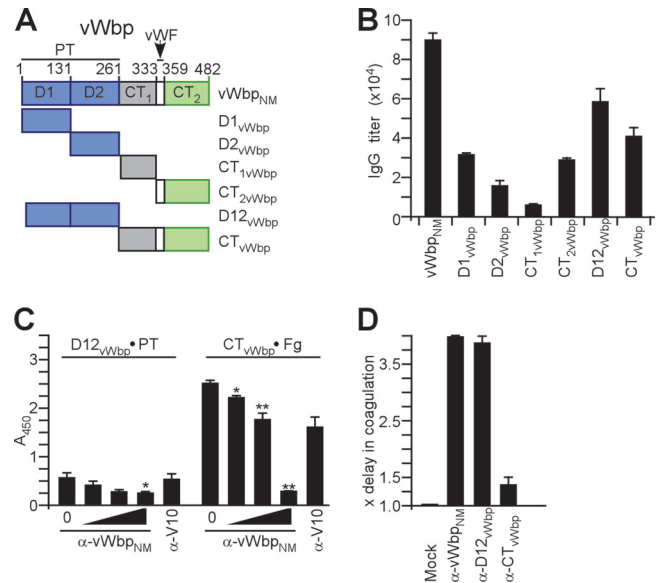

FIG 3 Immune responses to von Willebrand factor binding protein (vWbp). (A) Drawing to illustrate the primary structure of vWbp from *S. aureus* Newman ($vWbp_{NM}$), which was purified from *E. coli* via an N-terminal His_6 tag. $vWbp_{NM}$ encompasses the D1 and D2 domains, involved in prothrombin binding, as well as the von Willebrand factor (vWF) binding domain. In addition to $vWbp_{NM}$, the $D1_{vWbp}$, $D2_{vWbp}$, $D12_{vWbp}$, CT_{1vWbp}, CT_{2vWbp}, and CT_{vWbp} domains were purified. (B) Rabbits were immunized with purified $vWbp_{NM}$, and immune sera were examined by an ELISA for serum IgG reactive with $vWbp_{NM}$, $D1_{vWbp}$, $D2_{vWbp}$, $D12_{vWbp}$, CT_{1vWbp}, CT_{2vWbp}, and CT_{vWbp}. (C) The association of $D12_{vWbp}$ with human prothrombin or the binding of CT_{vWbp} to fibrinogen was measured by an ELISA and perturbed with increasing concentrations of rabbit IgG directed against $vWbp_{NM}$ (0 μM, 0.013 μM, 0.13 μM, or 1.3 μM) or the plague vaccine antigen V10 (1.3 μM) as a control. (D) Affinity-purified rabbit IgG specific for $vWbp_{NM}$ (α-$vWbp_{NM}$), $D12_{vWbp}$ (α-$D12_{vWbp}$), or CT_{vWbp} (α-CT_{vWbp}) was added to citrate-treated mouse blood and inoculated with *S. aureus* Newman to monitor the inhibition of staphylococcal coagulation.

and D). A minimal concentration of 1.3 μM anti-vWbp IgG blocked $D12_{vWbp}$ binding to prothrombin as well as the CT_{vWbp} association with fibrinogen (Fig. 3D).

Equimolar amounts of affinity-purified IgG were added to citrate-blood samples obtained from naïve BALB/c mice and subsequently inoculated with a *coa* mutant derived from *S. aureus* Newman (6). Compared to control samples without antibody, both anti-vWbp and anti-$D12_{vWbp}$ antibodies caused small delays in the clotting time, whereas anti-CT_{vWbp} antibody did not delay the clotting time (Fig. 3D). Thus, rabbits respond to immunization with $vWbp_{NM}$ by generating antigen-specific IgG directed against $D12_{vWbp}$, CT_{1vWbp}, and CT_{vWbp}. Antibodies against $D12_{vWbp}$ interfere with the vWbp-mediated coagulation of mouse blood *in vitro*.

Antibodies against vWbp domains and their protective effect on staphylococcal disease. Purified $vWbp_{NM}$, $D12_{vWbp}$, or CT_{vWbp} was emulsified and injected into BALB/c mice ($n = 10$) as a prime-boost regimen. Sera of mock (PBS)-immunized or $vWbp_{NM}$-, $D12_{vWbp}$-, and CT_{vWbp}-immunized animals were examined by an ELISA for IgG responses to antigen, revealing specific immune responses in vaccinated animals but not in control mice (Fig. 4A and B). Of note, the immunization of mice with $vWbp_{NM}$ raised antibodies predominantly against $D12_{vWbp}$ and, to a lesser degree, antibodies that were directed against CT_{vWbp} (Fig. 4A). $D12_{vWbp}$
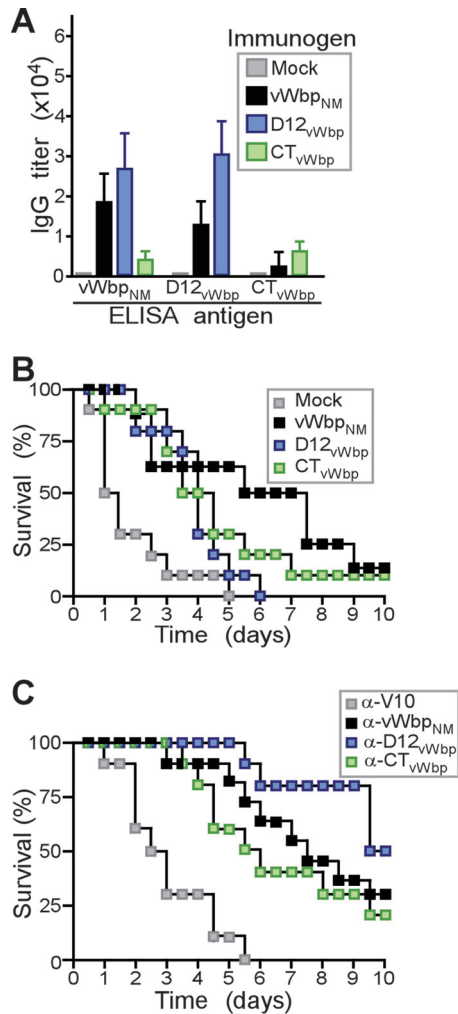
FIG 4 von Willebrand factor binding protein (vWbp) domains as vaccine antigens. (A) Recombinant purified vWbp$_{NM}$, D12$_{vWbp}$, and CT$_{vWbp}$ were used to immunize BALB/c mice ($n = 5$) with a prime-boost regimen, and immune sera were analyzed by an ELISA for the reactivity of mouse serum IgG toward purified vWbp$_{NM}$, D12$_{vWbp}$, and CT$_{vWbp}$. (B) Cohorts of BALB/c mice ($n = 10$) were immunized with a prime-boost regimen of purified vWbp$_{NM}$, D12$_{vWbp}$, and CT$_{vWbp}$ and challenged by intravenous injection with *S. aureus* Newman (1×10^8 CFU). The survival of animals was monitored over 10 days. (C) Affinity-purified rabbit IgG specific for vWbp$_{NM}$ (α-vWbp$_{NM}$), D12$_{vWbp}$ (α-D12$_{vWbp}$), CT$_{vWbp}$ (α-CT$_{vWbp}$), or V10 (α-V10) was injected at a concentration of 5 mg/kg of body weight into the peritoneal cavity of naïve BALB/c mice. Passively immunized mice were challenged by intravenous injection with *S. aureus* Newman (1×10^8 CFU), and the survival of animals was monitored over 10 days.
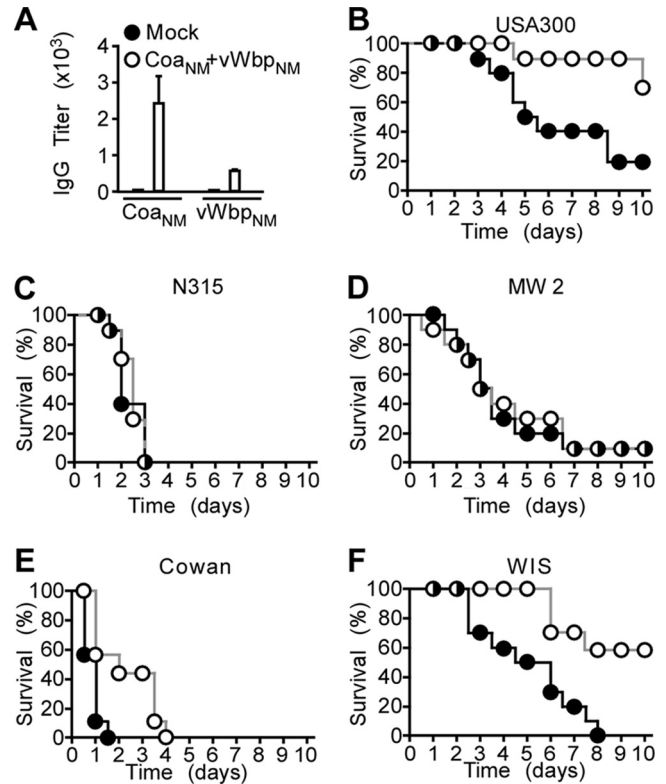

FIG 5 Immunization of mice with the Coa$_{NM}$/vWbp$_{NM}$ vaccine and spectrum of disease protection against different *S. aureus* isolates. (A) Recombinant Coa$_{NM}$/vWbp$_{NM}$ or mock (PBS) vaccine was used to immunize BALB/c mice ($n = 5$) with a prime-boost regimen. Immune sera were analyzed by an ELISA for the reactivity of mouse serum IgG toward purified Coa$_{NM}$ and vWbp$_{NM}$. (B to F) Cohorts of BALB/c mice ($n = 10$) were immunized with a prime-boost regimen of purified Coa$_{NM}$/vWbp$_{NM}$ or mock vaccine and challenged by intravenous injection with *S. aureus* USA300 (B), N315 (C), MW2 (D), CowanI (E), or WIS (F). The survival of animals was monitored over 10 days.

immunization raised high-titer antibodies that reacted with full-length vWbp$_{NM}$ (Fig. 4A). In contrast, CT$_{vWbp}$ immunization generated weak antibody responses (Fig. 4A). Mice were challenged by intravenous injection with *S. aureus* Newman, and a 10-day observation period was used to assess protection against lethal sepsis (Fig. 4B). Compared to mock-immunized animals, vaccination with vWbp$_{NM}$, D12$_{vWbp}$, or CT$_{vWbp}$ resulted in an increased time to death ($P < 0.01$ for vWbp$_{NM}$ versus PBS, $P < 0.05$ for D12$_{vWbp}$ versus PBS, and $P < 0.05$ for CT$_{vWbp}$ versus PBS). Immune responses against vWbp$_{NM}$ outperformed vaccination with D12$_{vWbp}$ but not CT$_{vWbp}$ in generating protection

against lethal *S. aureus* challenge ($P < 0.05$ for vWbp$_{NM}$ versus D12$_{vWbp}$ and $P > 0.05$ for vWbp$_{NM}$ versus CT$_{vWbp}$) (Fig. 4B).

We asked whether antibodies directed against D12$_{vWbp}$ or CT$_{vWbp}$ provide protection against a lethal *S. aureus* challenge. Affinity-purified rabbit IgG was injected into the peritoneal cavity of naïve BALB/c mice at a concentration of 5 mg/kg of body weight (Fig. 4C). Twenty-four hours later, animals were challenged by the intravenous injection of *S. aureus* Newman (Fig. 4C). Compared to control antibodies (anti-V10 antibody), IgGs directed against vWbp$_{NM}$, D12$_{vWbp}$, or CT$_{vWbp}$ each caused a delay in the time to death for the corresponding cohort of challenged animals ($P < 0.05$ for all vaccines versus anti-V10 antibody) (Fig. 4C). No significant differences in disease protection were detected among antibodies directed against D12$_{vWbp}$, CT$_{vWbp}$, and full-length vWbp$_{NM}$ (Fig. 4C). Thus, in contrast to D12$_{vWbp}$, immunization with the CT$_{vWbp}$ domain elicits low antibody responses. The passive transfer of antibodies against D12$_{vWbp}$ and CT$_{vWbp}$ provides similar levels of protection against a lethal *S. aureus* Newman challenge. These data suggest that the antibody-mediated neutralization of *S. aureus* Newman vWbp via antibodies directed against either D12$_{vWbp}$ or CT$_{vWbp}$ correlates with disease protection. Following exposure to full-length vWbp$_{NM}$, BALB/c mice mounted robust immune responses against D12$_{vWbp}$ and CT$_{1vWbp}$ but generated few antibodies against CT$_{vWbp}$.
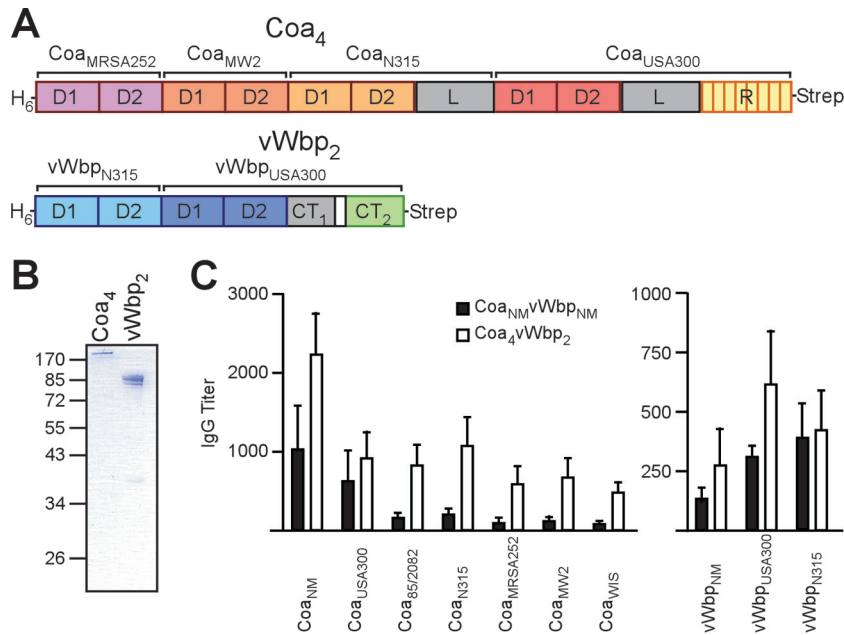
FIG 6 Immunogenicity of the Coa₄/vWbp₂ vaccine. (A) Drawing to illustrate the design of the Coa₄ and vWbp₂ vaccine components. Coa₄ is comprised of an N-terminal six-histidyl tag (H_6); the Coa D12 domains of *S. aureus* strains MRSA252, MW2, and N315; and the full-length mature sequence of Coa from strain USA300 in addition to a C-terminal Strep tag. vWbp₂ is comprised of an N-terminal six-histidyl tag, the vWbp D12 domains of *S. aureus* N315, and the full-length mature sequence of vWbp from strain USA300 in addition to a C-terminal Strep tag. (B) Coa₄ and vWbp₂ were purified from *E. coli* via streptavidin affinity chromatography and analyzed by Coomassie-stained SDS-PAGE gels. (C) BALB/c mice ($n = 5$) were immunized with either Coa$_{NM}$/vWbp$_{NM}$ or Coa₄/vWbp₂ by using a prime-boost regimen. Immune sera were analyzed by an ELISA for the reactivity of mouse serum IgG toward purified recombinant coagulase from *S. aureus* Newman (Coa$_{NM}$), USA300 (Coa$_{NM}$), 85/2082 (Coa$_{85/2082}$), N315 (Coa$_{N315}$), MRSA252 (Coa$_{MRSA252}$), MW2 (Coa$_{MW2}$), or WIS (Coa$_{WIS}$) as well as purified recombinant vWbp from *S. aureus* Newman (vWbp$_{NM}$), USA300 (vWbp$_{NM}$), or N315 (vWbp$_{N315}$).

Cross-protective attributes of the Coa$_{NM}$/vWbp$_{NM}$ vaccine. Purified recombinant Coa$_{NM}$ and vWbp$_{NM}$ were emulsified and injected into BALB/c mice ($n = 10$) as a prime-boost immunization regimen. Sera of mock (PBS)- and Coa$_{NM}$/vWbp$_{NM}$-immunized animals were examined by an ELISA for IgG responses to each antigen (Fig. 5A). Mice were challenged by the intravenous injection of *S. aureus* and monitored for 10 days (Fig. 5). Coa$_{NM}$/vWbp$_{NM}$ immunization raised protection against *S. aureus* USA300 (CC8, the same type as *S. aureus* Newman) but not against MW2 (CC1) or N315 (CC5) (Fig. 5B to D). Nevertheless, Coa$_{NM}$/vWbp$_{NM}$ immunization generated protection against challenge with *S. aureus* CowanI (CC30) and WIS (CC45) (Fig. 5E and F). Taken together, these data indicate that the Coa$_{NM}$/vWbp$_{NM}$ vaccine provided type-specific immunity (CC8 strains Newman and USA300) as well as cross-protection against some (CC30 and CC45), but not all, coagulase-type strains (CC1 and CC5).

Immune responses elicited by the Coa₄/vWbp₂ vaccine. A polypeptide that harbors the D12 domains of Coa$_{MRSA252}$, Coa$_{MW2}$, and Coa$_{N315}$ and full-length Coa$_{USA300}$ in addition to N-terminal His₆ and C-terminal Strep tags (Coa₄) was engineered (Fig. 6A). Coa₄ was purified by affinity chromatography on Strep-Tactin-Sepharose and Ni-NTA Sepharose (Fig. 6B). When analyzed by Coomassie-stained SDS-PAGE gels, affinity-purified Coa₄ was revealed as a 190-kDa polypeptide (Fig. 6B). Coa₄ encompasses the D12 domains from four of the most frequently detected coagulase-type *S. aureus* isolates from North American patients (CC1, CC5, CC8, and CC30) (9). The vWbp₂ polypeptide encompasses the D12 domain of vWbp$_{N315}$ and full-length vWbp$_{USA300}$ in addition to N-terminal His₆ and C-terminal Strep tags

(Fig. 6A). vWbp₂ was purified by affinity chromatography and migrated at the expected mass of 85 kDa on Coomassie-stained SDS-PAGE gels (Fig. 6B). Mice ($n = 5$) were immunized with a prime-boost regimen of Coa$_{NM}$/vWbp$_{NM}$ or Coa₄/vWbp₂, and immune responses to various coagulase and von Willebrand factor binding protein types were examined by an ELISA (Fig. 6C and D). The Coa$_{NM}$/vWbp$_{NM}$ vaccine raised antibodies in mice that bound to the coagulases from CC8 strains but displayed little cross-reactivity toward Coa$_{N315}$, Coa$_{MRSA252}$, Coa$_{MW2}$, or Coa$_{WIS}$. In comparison, Coa₄ immunization raised high-titer antibodies not only against CC8 type coagulases but also against the coagulases from CC1, CC5, CC30, and CC45 strains. Compared to vWbp$_{NM}$, vWbp₂ raised high-titer antibodies against vWbp of the CC5 and CC8 strains (Fig. 6D).

Cross-protective attributes of the Coa₄/vWbp₂ vaccine. Purified recombinant Coa₄/vWbp₂ was emulsified and injected into BALB/c mice ($n = 10$) by using a prime-boost immunization regimen. Sera of mock (PBS)- and Coa₄/vWbp₂-immunized animals were examined by an ELISA for IgG responses to Coa₄ as well as vWbp₂, which revealed antigen-specific immune responses in vaccinated but not in control mice (Fig. 7A). The intravenous injection of mice with *S. aureus* and a 10-day observation period were used to assess vaccine protection against lethal challenge with various strains (Fig. 7). As expected, Coa₄/vWbp₂ immunization raised protection against *S. aureus* CC8 strain USA300 (6). Similar to Coa$_{NM}$/vWbp$_{NM}$ immunization, the Coa₄/vWbp₂ vaccine raised protection against *S. aureus* CowanI (CC30) and WIS (CC45) challenges. Unlike Coa$_{NM}$/vWbp$_{NM}$, Coa₄/vWbp₂ protected mice against lethal challenge with either *S. aureus* N315
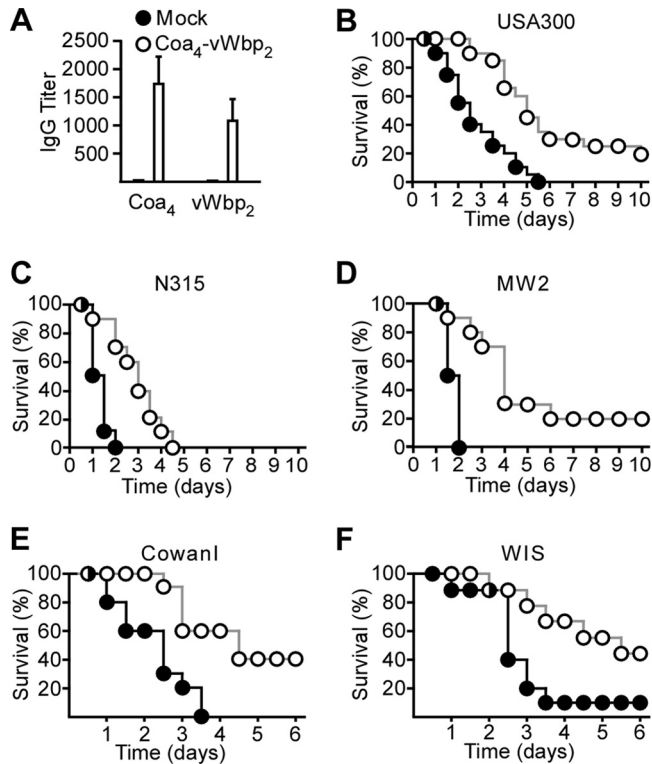
FIG 7 Immunization of mice with the Coa$_4$/vWbp$_2$ vaccine and spectrum of disease protection against different *S. aureus* isolates. (A) Coa$_4$/vWbp$_2$ or mock (PBS) vaccine was used to immunize BALB/c mice (*n* = 5) with a prime-boost regimen. Immune sera were analyzed by an ELISA for the reactivity of mouse serum IgG toward purified Coa$_4$ and vWbp$_2$. (B to F) Cohorts of BALB/c mice (*n* = 20 in panel B and *n* = 10 in panels C to F) were immunized with a prime-boost regimen of purified Coa$_4$/vWbp$_2$ or mock vaccine (B) and challenged by intravenous injection with *S. aureus* USA300 (B), N315 (C), MW2 (D), CowanI (E), or WIS (F). The survival of animals was monitored over 10 days.

(CC5) or MW2 (CC1) (Fig. 7B to F) (*P* < 0.05 for mock versus Coa$_4$/vWbp$_2$). Taken together, these data indicate that the Coa$_{NM}$/vWbp$_{NM}$ vaccine provided type-specific immunity as well as cross-protection against some, but not all, coagulase-type strains (Fig. 5E and F). Furthermore, the Coa$_4$/vWbp$_2$ vaccine protected animals against a challenge with the relevant *S. aureus* CC types isolated from North American patients with staphylococcal disease (Fig. 7).

We asked whether Coa$_4$/vWbp$_2$ immunization protects mice against staphylococcal abscess formation. BALB/c mice were immunized with a prime-boost regimen of Coa$_4$/vWbp$_2$ or the mock control and challenged by the intravenous inoculation of a sublethal dose of *S. aureus* strain USA300, N315, MW2, or CowanI. Five days after challenge, animals were euthanized and necropsied, and kidneys were removed. One of the two kidneys from each mouse was fixed, thin sectioned, and stained with hematoxylin-eosin for subsequent histopathology analyses (Table 2). The other kidney was homogenized and spread onto agar plates to enumerate the staphylococcal load as CFU (Table 2). Coa$_4$/vWbp$_2$ immunization led to a significant reduction in the bacterial load for animals challenged with *S. aureus* MW2 and CowanI but not for mice challenged with USA300 and N315. Of note, Coa- or vWbp-specific antibodies do not promote the opsonophagocytic killing

of bacteria but interfere with staphylococcal abscess formation by neutralizing coagulases, thereby reducing the ability of staphylococci to replicate within the protective environment of these lesions (10). Compared to mock-immunized animals, Coa$_4$/vWbp$_2$ immunization reduced staphylococcal abscess formation in renal tissues 5 days following challenge with *S. aureus* strain USA300, CowanI, MW2, or N315 (Table 2).

DISCUSSION

Early work on coagulase demonstrated that following *S. aureus* infection, humans as well as animals generate Coa-specific antibodies (25, 41). When transferred to naïve rabbits, these antibodies may neutralize *S. aureus* coagulation and, at least in some cases, may confer immunity to challenge with *S. aureus* (24, 26). The active immunization of rabbits with preparations containing coagulase prolonged the life of rabbits that had been challenged by intravenous inoculation with lethal doses of *S. aureus* (4). A comparison of different (phage-typed) *S. aureus* isolates for the inhibition of plasma clotting by coagulase antiserum revealed both phage type-specific and nonspecific neutralization (10, 15, 25, 26, 37). These data supported a general concept for the existence of serological types of Coa, which are not strictly linked to *S. aureus* phage types (38).

Purified coagulase toxoid, encompassing purified Coa from *S. aureus* strains M1 and Newman adsorbed onto aluminum phosphate, was examined for the therapeutic immunization of 71 patients with chronic furunculosis (14). Compared to placebo, coagulase immunization generated an increase in coagulase-specific antibody titers but failed to improve the clinical outcome of chronic furunculosis (14). Of note, the development of neutralizing antibodies or the possibility of type-specific immunity was not examined (14). Thus, although early work revealed the preclinical efficacy of coagulase subunit vaccines, clinical studies failed to demonstrate efficacy in a human trial. As most of those studies were conducted from 1945 to 1965, one must consider the limited tools for the isolation of highly purified coagulases as well as the inability to type *S. aureus* strains or coagulase vaccine preparations on the basis of their nucleotide sequence. Furthermore, earlier studies were conducted without a knowledge of vWbp or of the molecular mechanisms of Coa- and vWbp-mediated prothrombin activation and fibrinogen cleavage (12, 21). We recently observed that both coagulases secreted by *S. aureus* Newman, Coa$_{NM}$ and vWbp$_{NM}$, are sufficient for the ability of this strain to cause abscess formation and rapidly lethal bacteremia in mice (6). In active and passive immunization experiments, antibodies against both Coa$_{NM}$ and vWbp$_{NM}$ were required to confer protection against abscess formation or lethal bacteremia (6). On the basis of those observations, we hypothesize that coagulases may function as protective antigens that elicit antibody responses against Coa and vWbp, which protect animals and humans against *S. aureus* disease (6). In agreement with this model, the expression of *coa* and *vwb* is a universal trait of *S. aureus* strains (5). Of note, the *coa* gene of *S. aureus* isolates is variable (29), with a greater variation in amino acid sequence than even the tandem repeats of the protein A (*spa*) gene; the variation in *spa* is used for epidemiological typing experiments (20, 43). *S. aureus* mutants that are unable to express *coa* have not yet been isolated from humans with manifest staphylococcal disease. The *vwb* gene is less variable (29). By analyzing currently available *S. aureus* genome sequences for *vwb* homology, we identified three alleles. Two of the *vwb* alleles varied in

TABLE 2 Active immunization of mice with $Coa_4/vWbp_2$ and protection against challenge with *S. aureus* strain USA300, N315, MW2, or CowanI[f]

Vaccine	Staphylococcal load in renal tissue			Abscess formation	
	Mean $\log_{10}$ CFU $\cdot$ g^{-1} (SEM)[a]	P value[b]	Reduction ($\log_{10}$ CFU $\cdot$ g^{-1})[c]	Mean no. of lesions (SEM)[d]	P value[e]
S. aureus USA300					
Mock	7.31 (0.37)	NA[g]	NA	8.8 (1.72)	
$Coa_4/vWbp_2$	6.48 (0.41)	0.150	0.835	4.3 (1.11)	0.0434
S. aureus N315					
Mock	7.25 (0.13)	NA	NA	16.6 (1.49)	
$Coa_4/vWbp_2$	7.10 (0.24)	0.805	0.151	11.3 (0.84)	0.0205
S. aureus MW2					
Mock	8.04 (0.25)	NA	NA	66.5 (8.41)	
$Coa_4/vWbp_2$	7.25 (0.20)	0.029	0.789	27.5 (4.39)	0.0011
S. aureus CowanI					
Mock	6.94 (0.16)	NA	NA	7.9 (1.27)	
Coa4/vWbp2	5.59 (0.51)	0.028	1.35	4.6 (0.73)	0.0279

[a] Means of staphylococcal load calculated as $\log_{10}$ CFU $\cdot$ g^{-1} in homogenized renal tissues 5 days after infection of cohorts of 10 BALB/c mice per immunization. Standard errors of the means are indicated.

[b] Statistical significance was calculated with the Mann-Whitney test, and P values were recorded; P values of <0.05 were deemed significant.

[c] Reduction in bacterial load calculated as $\log_{10}$ CFU $\cdot$ g^{-1}.

[d] Histopathology of thin-sectioned, hematoxylin-eosin-stained kidneys from 8 to 10 animals. The average number of abscesses per kidney was recorded and averaged again for the final mean (and standard error of the mean).

[e] Statistical significance was calculated with the nonparametric Mann-Whitney test, and P values were recorded. P values of <0.05 are significant.

[f] BALB/c mice ($n = 8$ to 10) were challenged by the intravenous inoculation of 5×10^6 CFU *S. aureus* USA300, 1×10^7 CFU *S. aureus* N315, 2×10^7 CFU *S. aureus* MW2, or 1×10^8 CFU *S. aureus* CowanI. Five days later, animals were sacrificed by CO_2 asphyxiation, and both kidneys were removed. One kidney was fixed in formaldehyde, embedded in paraffin, thin sectioned, and hematoxylin-eosin stained, and four sagittal sections per kidney were analyzed for abscess formation. The other kidney was homogenized in PBS containing 1% Triton X-100, the homogenate was spread onto agar medium for colony formation, and the staphylococcal load was enumerated as CFU. The experiment was conducted twice.

[g] NA, not applicable.

their coding sequences for the D12 domain (*S. aureus* N315 and USA300 are representatives of these alleles), whereas the third allele harbored a nucleotide substitution positioning a premature stop codon at position 317 in the D12 domain (*S. aureus* MRSA252).

Enabled by these observations, we report here that the immunization of rabbits or mice with Coa and vWbp generated antibodies predominantly against the D12 domain of Coa_{NM} or $vWbp_{NM}$. D12-specific antibodies neutralized the coagulase activities of *S. aureus* Newman and, when transferred to naïve animals, conferred protection against lethal bacteremia. The neutralization and disease protection of Coa_{NM}- and $vWbp_{NM}$-specific antibodies occurred in a type-specific manner, not unlike the type-specific immunity reported previously for *Streptococcus pyogenes* M proteins (22, 23) or the pilus (T) antigens of *S. pyogenes* and *Streptococcus agalactiae* (30, 32). Informed by the structural vaccinology approach for pilus antigens (32, 39), we engineered two polypeptides that encompass the D12 domains of the major Coa and vWbp types from the North American *S. aureus* isolates: CC1, CC5, CC8, CC30, and CC45 strains (42). The purified products Coa_4 and $vWbp_2$ were used as antigens and elicited antibody responses against the D12 domains of every Coa and vWbp type examined. The immunization of mice with $Coa_4/vWbp_2$ provided protection against lethal bacteremia challenge with representative *S. aureus* CC1, CC5, CC8, CC30, and CC45 strains. Thus, the design criteria for the $Coa_4/vWbp_2$ vaccine, to generate broad-coverage immune responses to Coa and vWbp against clinically relevant *S. aureus* strains, have been met.

In addition to the type-specific neutralization of Coa and vWbp via antibodies directed against the D12 domain, antibodies against the R (Coa) and CT (vWbp) domains also provided protection against *S. aureus* disease. As antibodies against the R and CT domains do not affect the coagulation of fibrin via secreted Coa·prothrombin and vWbp·prothrombin complexes, we surmise that these adaptive immune mechanisms target coagulases via another mechanism. We currently do not appreciate how antibodies against the R domain of Coa or the CT domain of vWbp provide protection. It seems plausible that these antibodies may mediate the removal of Coa and vWbp from circulation via the binding of immune complexes to Fc receptors on macrophages. Until the molecular mechanism of protection is revealed, the overall value of a vaccine strategy that targets the R and CT domains of Coa and vWbp cannot be appreciated.

ACKNOWLEDGMENTS

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