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RESEARCH ARTICLE

Toward Optimization of a Rabbit Model of *Staphylococcus aureus* (USA300) Skin and Soft Tissue Infection

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ABSTRACT *Staphylococcus aureus* remains a leading cause of skin and soft tissue infections (SSTIs) globally. In the United States, many of these infections are caused by isolates classified as USA300. Our understanding of the success of USA300 as a human pathogen is due in part to data obtained from animal infection models, including rabbit SSTI models. These animal models have been used to study *S. aureus* virulence and pathogenesis and to gain an enhanced understanding of the host response to infection. Although significant knowledge has been gained, the need to use a relatively high inoculum of USA300 (1×10^8 to 5×10^8 CFU) is a caveat of these infection models. As a step toward addressing this issue, we created mutations in USA300 that mimic those found in *S. aureus* strains with naturally occurring rabbit tropism—namely, single nucleotide polymorphisms in *dltB* and/or deletion of *rot*. We then developed a rabbit SSTI model that utilizes an inoculum of 10^6 USA300 CFU to cause reproducible disease and tested whether primary SSTI protects rabbits against severe reinfection caused by the same strain. Although there was modest protection against severe reinfection, primary infection and reinfection with rabbit-tropic USA300 strains failed to increase the overall level of circulating anti-*S. aureus* antibodies significantly. These findings provide additional insight into the host response to *S. aureus*. More work is needed to further develop a low-inoculum infection model that can be used to better test the potential of new therapeutics or vaccine target antigens.

IMPORTANCE Animal models of *S. aureus* infection are important for evaluating bacterial pathogenesis and host immune responses. These animal infection models are often used as an initial step in the testing of vaccine antigens and new therapeutics. The extent to which animal models of *S. aureus* infection approximate human infections remains a significant consideration for translation of results to human clinical trials. Although significant progress has been made with rabbit models of *S. aureus* infection, one concern is the high inoculum needed to cause reproducible disease. Here, we generated USA300 strains that have tropism for rabbits and developed a rabbit SSTI model that uses fewer CFU than previous models.

KEYWORDS rabbit, MRSA, USA300, abscess, recurrent infection, animal model, *Staphylococcus aureus*

Skin and soft tissue infections (SSTIs), including impetigo, cellulitis, folliculitis, furuncles, and carbuncles, are prevalent worldwide. In the United States, SSTIs requiring medical intervention accounted for approximately 4.8 episodes per 100 persons/year from 2005 to 2010 (1). *Staphylococcus aureus* is the leading cause of SSTIs in the United States, and hospitalization-related costs for these infections average ~$4.3 billion per year (2). A methicillin-resistant

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S. aureus (MRSA) clone known as USA300 emerged in the early 2000s and rapidly became the most prevalent cause of community-associated infections, largely SSTIs (3–7). USA300 remains the most common cause of *S. aureus* SSTIs in the United States (8, 9), and there is a relatively high rate of recurring infection (15% to 70%) (10–12). For example, Miller et al. reported that 51% of patients treated for *S. aureus* skin infection developed recurrent infection within 6 months (13). Multiple factors and comorbidities, such as age, diabetes, exposure to health care settings, and/or history of SSTIs, potentially predispose a person to SSTIs (11, 14). Nonetheless, the host immune response and pathogen factors that contribute to recurrent SSTIs are incompletely understood.

Animal models that approximate human disease are important for enhancing our knowledge of host-pathogen interactions. This knowledge includes a better understanding of the role that pathogen virulence factors play in disease progression, as well as a detailed assessment of the host immune response to the invading microbe. *S. aureus* is a ubiquitous pathogen capable of infecting several different mammalian species. Following the interspecies host jumps, bacterial populations adapt to the new host environment, and they can develop a tropism and an enhanced fitness for the new host (15). The rabbit has been used to study *S. aureus* pathogenesis for many decades (16–19), and some *S. aureus* strains have a natural tropism for this species. Such adaptation implies that a more physiological inoculum is required to cause infection. One such *S. aureus* strain, classified as ST121, emerged as an epidemic rabbit clone that causes skin abscesses and mastitis in commercial rabbitries (20, 21). The rabbit-tropic ST121 clone, as well as rabbit ST1 and ST96 clones, have single nucleotide polymorphisms (SNPs) in *dltB* (encoding DltB) compared with human ST121 clinical isolates. DltB is an O-acyltransferase that catalyzes incorporation of D-alanine (D-Ala) into lipoteichoic and wall teichoic acids of the *S. aureus* cell wall (22, 23). Positively charged D-alanyl ester residues decrease the negative net charge of the bacterial surface, which leads to increased resistance to antibiotics and antimicrobial peptides (22, 24–26). Although the exact contribution of DltB to *S. aureus* infection in rabbits is not known, the observed high level of *dltB* polymorphisms among rabbit *S. aureus* isolates suggests it is important for host adaptation (21). Amino acid variance within DltB has been reported among the most prevalent rabbit *S. aureus* lineages, including T113K, Y250H, and *405Y in ST121 isolates, I2T and *405Q in ST1, and K402R and *405Q in ST96 (21, 27). In addition to *dltB* polymorphisms, the ST121 clone has a nonsense mutation in *rot*, which encodes a transcription factor known as repressor of toxins (Rot) (21). Rot is a DNA binding protein that regulates numerous virulence factors and has been shown to downregulate various secreted toxins and proteases, as well as to upregulate cell surface adhesins (28, 29). Therefore, inactivation or deletion of *rot* could lead to increased tissue damage caused by alpha-hemolysin and other secreted proteases (28). Although human ST121 clinical isolates fail to cause significant disease in rabbits (21), engineering the *dltB* and *rot* mutations in these isolates creates strains that cause reproducible skin disease in rabbits with a relatively low inoculum (21). It is currently not known why specific SNPs in the *dltB* gene render the pathogen more infectious in rabbits.

Many of our current *S. aureus* SSTI models in rabbits require a relatively high inoculum, such as 10^8 CFU or greater, to cause reproducible disease. The large bolus of bacteria needed to cause measurable (and reproducible) disease is a caveat of these animal infection models, as such a large inoculum is presumably not needed for human skin infections. Thus, the extent to which these animal infection models recapitulate human SSTIs could be brought into question. Moreover, a large inoculum might mask subtle differences in strain virulence and host responses or positive effects of candidate therapeutics and vaccines. As a step toward addressing this problem, we developed a rabbit model of USA300 SSTI that requires fewer bacteria to cause reproducible disease compared with previous rabbit USA300 skin infection models. We then used the low-inoculum animal infection model to evaluate the ability of primary SSTI caused by USA300 to protect against a subsequent infection with the same strain.

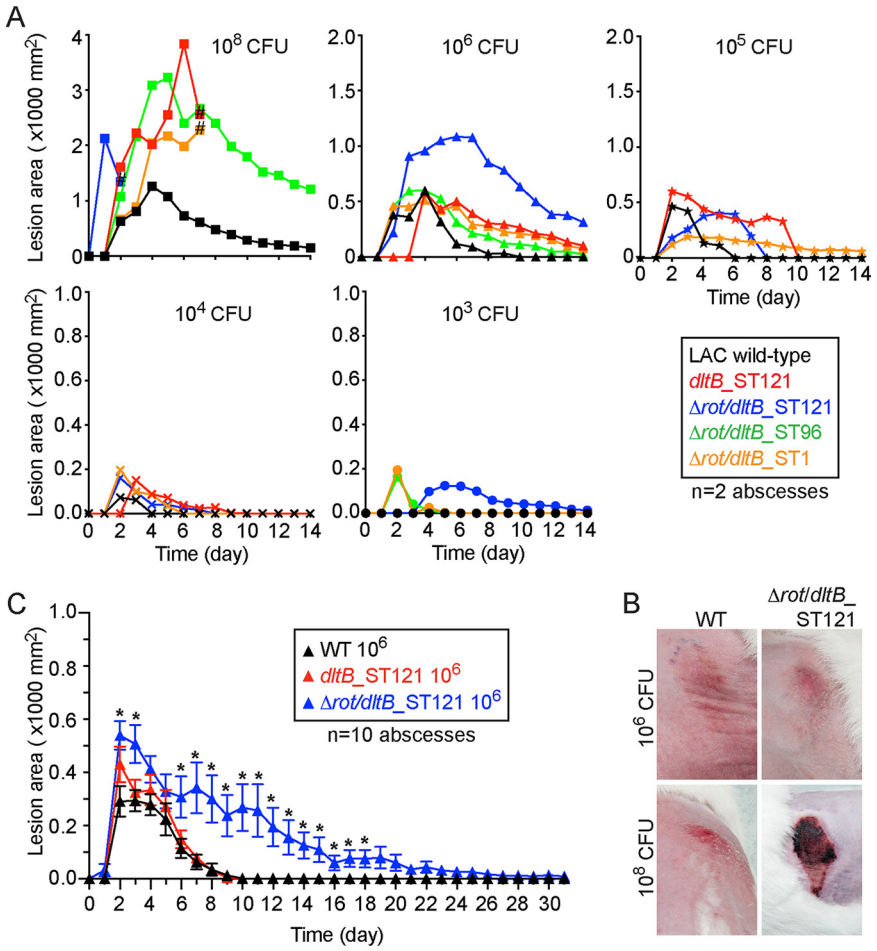

FIG 1 Mutation in *dltB* and deletion of *rot* increase rabbit tropism of USA300. (A) Pilot study to determine optimal inoculum (n = 1 rabbit per condition, 2 abscesses). (B) Gross pathology of skin lesions caused by 10^6 or 10^8 CFU of wild-type or Δ*rot*/*dltB*_ST121 mutant strains. Images are from a representative skin lesion. Blue dotted line visible on some images indicates the site of inoculation. (C) Large-scale experiment using the strains and inoculum determined in the pilot study (A). Skin lesions were measured daily, and abscess area (including dermonecrotic area) was calculated as described in Materials and Methods. #, Animals that met endpoint criteria early and were euthanized. *, $P < 0.05$ versus WT.

RESULTS

Targeted mutations in the *dltB* gene increase rabbit tropism of USA300. Rabbit models of *S. aureus* skin infection have been used widely to evaluate strain virulence and pathogenesis. As with rodent *S. aureus* infection models, a relatively large bolus of USA300 (~1×10^8 to 5×10^8 CFU or greater) is needed to cause reproducible skin disease in rabbits. To develop a rabbit model of USA300 skin infection that requires fewer bacteria to cause reproducible disease, we created USA300 isogenic strains with the *dltB* and/or *rot* mutations present in rabbit *S. aureus* isolates (see Fig. S1 and S2 in the supplemental material). Mutations created in the USA300 *dltB* gene resulted in the following amino acid substitutions: I2T and *405Q, mimicking DltB_ST1; K402R and *405Q, mimicking DltB_ST96; and T113K, Y250H and *405Y, mimicking DltB_ST121. The USA300 wild-type (WT) and isogenic mutant strains had comparable growth *in vitro* (Fig. S3).

We next performed a pilot study to identify the minimum subcutaneous (s.c.) inoculum (10^3 to 10^8 CFU) of each USA300 strain that causes reproducible abscesses in rabbits (Fig. 1A). Inoculation of rabbits with *S. aureus dltB* and *rot* mutant strains resulted in larger and/or more severe skin lesions than those from the USA300 wild-type strain, especially at the highest inoculum tested (10^8 CFU) (Fig. 1A and B and Fig. S4).

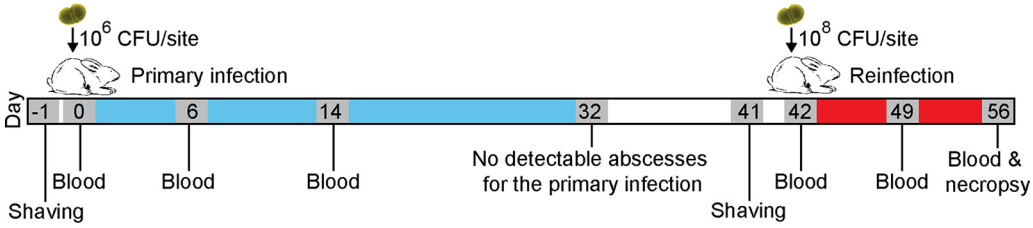

FIG 2 Schematic of the infection/reinfection study in rabbits. Five animals per strain were inoculated s.c. with 10^6 CFU/site into right and left flanks for the primary infection. One week after no palpable abscesses were detected, animals were reinfected with a dose of 10^8 CFU/site of the same strain. Skin lesions following reinfection were monitored for 14 days postinoculation. Blood was obtained on the days indicated.

Moreover, skin lesions of animals that received 10^8 CFU of USA300Δ*rot/dltB*_ST1, USA300*dltB*_ST121, or USA300Δ*rot/dltB*_ST121 had more dermonecrosis than those of rabbits infected with the wild-type parent strain (Fig. 1B and Fig. S4). By comparison, 10^3 CFU of USA300Δ*rot/dltB*_ST121 caused an acute inflammatory response in the vicinity of the inoculation site, but only one abscess formed (of 2 sites inoculated) and it resolved by day 10 (Fig. 1A). Rabbits inoculated with 10^5 CFU in each flank developed measurable abscesses that persisted for several days, and abscesses caused by the wild-type strain resolved more rapidly (Fig. 1A). Nonetheless, within the first 48 h after inoculation it was difficult to delineate abscess formation and the acute inflammatory response in rabbits infected with 10^3, 10^4, or 10^5 CFU per flank. By comparison, animals inoculated with 10^6 CFU developed skin lesions that were reproducible and measured readily (Fig. 1A and B). Therefore, we selected 10^6 CFU as the inoculum to use for comparison of wild-type and mutant strains in a large-scale experiment (Fig. 1C). Notably, rabbits infected with USA300Δ*rot/dltB*_ST121 developed skin abscesses/lesions that were significantly larger than those caused by the wild-type strain and took longer to resolve (on average it took ~7 days for the wild-type strain versus ~8 days for *dltB*_ST121 and ~17 days for Δ*rot/dltB*_ST121 mutant strains) (Fig. 1C). These findings highlight the rabbit tropism of a human USA300 strain that has been engineered to contain SNPs found in rabbit *S. aureus* isolates.

Rabbit model of *S. aureus* recurrent SSTI. We next developed a rabbit model of infection/reinfection that utilized the rabbit-tropic USA300 strains described above. For the primary infection, animals ($n = 5$) were inoculated subcutaneously (s.c.) with 10^6 CFU of either USA300 wild-type, USA300Δ*rot/dltB*_ST121, or USA300*dltB*_ST121 strains into the left and right flank (10 abscesses per infecting strain) (Fig. 2). To test the ability of primary infection to elicit protective immunity, a second infection with the same strain was performed following resolution of the first infection (Fig. 2). Animals were challenged with 10^8 CFU per flank for the secondary infection, since this inoculum caused severe skin lesions and/or led to early endpoints (euthanasia) in the pilot experiment regardless of the strain variant (Fig. 1A and Fig. 3). Primary infection conferred moderate protection against severe disease caused by reinfection, most notably for USA300Δ*rot/dltB*_ST121, or USA300*dltB*_ST121 strains (Fig. 3). In the pilot experiment, the average abscess size 4 days after infection with 10^8 CFU of USA300*dltB*_ST121 was ~20 cm^2 (Fig. 1A and Fig. 3B), whereas the average abscess size on the same day after reinfection was ~12.7 cm^2 (Fig. 3).

Histopathologic analysis revealed that skin lesions caused by the second infection (reinfection) with Δ*rot* and/or *dltB* mutant strains were predominantly dermonecrotic in nature and by day 14 of reinfection resulted in keratinizing cysts (USA300*dltB*_ST121; 8/10) or remained as chronic ulcerative dermatitis (USA300Δ*rot/dltB*_ST121; 6/6) with varied degrees of inflammation of the panniculus muscle layer (Tables 1 and 2; Fig. 4). Keratinizing cysts often contained a necrotic core and reflect resolving ulceration or abscessation (Fig. 4C and E). The cysts were surrounded by varied degrees of granulation tissue and often had regions of keratinization along the roof and active inflammation

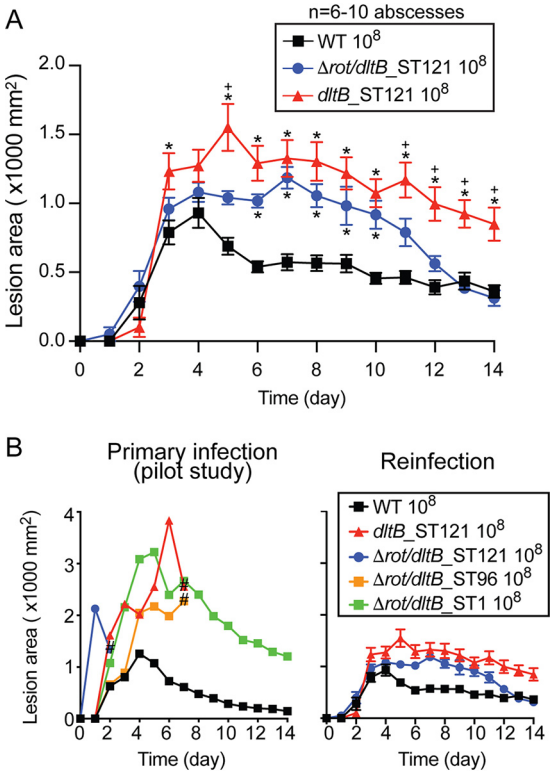

FIG 3 Primary USA300 skin infection confers partial protection from reinfection. (A) Rabbits were reinfected s.c. with 10^8 CFU 1 week after complete resolution of abscesses caused by primary infection with 10^6 CFU of the same strain. Skin lesion area was measured daily for 14 days. Results are the mean from 6 to 10 abscesses per strain. *, $P < 0.05$ versus WT; +, $P < 0.05$ versus Δ*rot/dltB*_ST121. (B) Comparison of primary infection with 10^8 CFU from the pilot study (Fig. 1A) with reinfection with 10^8 CFU at as shown in panel A. For direct comparison, the *y* axis of panel A was scaled to match that of the *y* axis of the data plot from the primary infection in panel B. #, Animals that met endpoint criteria early and were euthanized.

bounded by granulation tissue along the floor (Fig. 4C). By comparison, 7 out of 10 lesions caused by the USA300 wild-type strain were dermal abscesses (Table 1). The loss of pilosebaceous units was minimal to moderate for dermal abscesses and keratinizing cysts (Fig. 4B and C). However, loss of pilosebaceous units was generally complete for chronic ulcers and corresponded with more severe skin lesions (Fig. 4D to F, and Table 2).

Reinfection fails to elicit a significant increase in anti-*S. aureus* antibody. To test whether infection and/or reinfection with USA300 strains alters anti-*S. aureus* antibody levels in circulation, we used flow cytometry to measure anti-*S. aureus* antibody titers in rabbit blood before and after s.c. inoculation with USA300 strains (Fig. 5). As with humans, healthy rabbits have detectable anti-*S. aureus* antibodies in circulation (Fig. 5, day 0). Although there was a trend toward increased antibody titers following reinfection with USA300 WT and *dltB*_ST121 strains, it was not significant (Fig. 5, day 49 and day 56).

TABLE 1 Histopathology of the skin lesions on day 14 after secondary infection[a]

	No. of lesions/no. of inoculation sites by strain:		
Skin pathology	**WT**	***dltB*_ST121**	**Δ*rot/dltB*_ST121**
Abscess	7/10	2/10	0/6
Keratinizing cyst	2/10	8/10	1/6
Chronic ulcerative dermatitis	3/10	8/10	6/6
Serocellular crust	3/10	8/10	6/6

[a]All tissue samples were examined and scored by a board-certified veterinary pathologist. Skin lesions were scored as number present/number of inoculation sites.

TABLE 2 Type and severity of lesions by strain[a]

Histological feature	Avg pathology score by strain:		
	WT	*dltB*_ST121	Δ*rot/dltB*_ST121
Serocellular crust	1.1	3.2	2.7
Granulation tissue	2.8	5.0	4.0
Loss of pilosebaceous units	2.1	5.0	5.0
Perivasculitis, lymphoplasmacytic and histiocytic	1.6	1.0	1.0
Panniculitis	0.6	3.6	2.3

[a]All tissue samples were examined and scored by a board-certified veterinary pathologist. A severity score for each lesion was determined based on the following criteria: 0 = not present, 1 = mild, 3 = moderate, 5 = severe.

DISCUSSION

Animal models are used widely for infectious disease research. More specifically, murine and rabbit models have been essential for gaining an enhanced understanding of *S. aureus* pathogenesis. Although the mouse infection models are arguably more tractable than rabbit infection models, rabbits (but not mice) have natural susceptibility to some lineages of *S. aureus*. In addition, the morphology and physiology of rabbit skin are also considered more relevant to humans than the skin of the mouse (20, 30–32). One caveat of rabbit models of *S. aureus* SSTI is the need for a relatively high inoculum of human *S. aureus* strains to cause reproducible disease. As a step toward addressing this issue, we generated rabbit-tropic USA300 strains and tested them in a rabbit model of *S. aureus* SSTI.

For these studies, we modified the genome of the human USA300 epidemic clone to contain naturally occurring *dltB* SNPs present in rabbit *S. aureus* isolates. These *dltB* SNPs increased rabbit tropism of USA300 such that a typical inoculum of 5×10^8 CFU with the WT strain (33) could be reduced to 10^6 CFU using the mutant strains and achieve reproducible skin disease (Fig. 1). Recently, Muñoz-Silvestre and colleagues described a rabbit intradermal model of *S. aureus* SSTI that used 3×10^2 CFU of a human ST121 strain containing rabbit-specific SNPs (34). Our rabbit infection model required a higher inoculum than that of Muñoz-Silvestre et al. This difference might be explained by the use of different *S. aureus* lineages in these model systems (e.g., ST8 versus ST121) (35). Indeed, there is significant variance in the inoculum needed even among different rabbit *S. aureus* lineages, likely a reflection of strain-dependent interactions with the host immune system (36). The site of inoculation also plays a crucial role in the initial recognition and host immune response to infection, and our use of s.c. (versus intradermal) inoculation could have contributed to differences in rabbit inocula in these studies (37, 38). This all said, the USA300 inoculum needed was reduced significantly by the rabbit tropism conferred by SNPs, and we used this lower-inoculum infection model to evaluate development of protection against severe disease in an infection/reinfection study.

Although *S. aureus* nasal colonization is a risk factor for nosocomial infection in susceptible individuals (39, 40), mortality associated with *S. aureus* bacteremia is reduced significantly in patients colonized by *S. aureus* (41). One likely explanation for the reduced mortality in these patients is the development of protection against severe *S. aureus* infection as a result of colonization and/or recurrent infections. Indeed, studies in mice have demonstrated that adaptive immunity is involved in protection against recurrent *S. aureus* SSTIs, which is comprised of a T-cell-mediated response in conjunction with anti-*S. aureus* antibody (42). To gain further insight into the basis of this protection, we used our rabbit *S. aureus* infection model to test whether reinfection caused an increase in circulating anti-*S. aureus* antibodies (Fig. 5). Notably, there was no significant increase in anti-*S. aureus* antibodies for up to 2 weeks after reinfection with the same strain. The lack of significant increase in anti-*S. aureus* antibody is likely explained by the preexistence of naturally occurring antibodies in rabbits (Fig. 5). That is, the rabbits had anti-*S. aureus* antibodies in circulation before the start of the

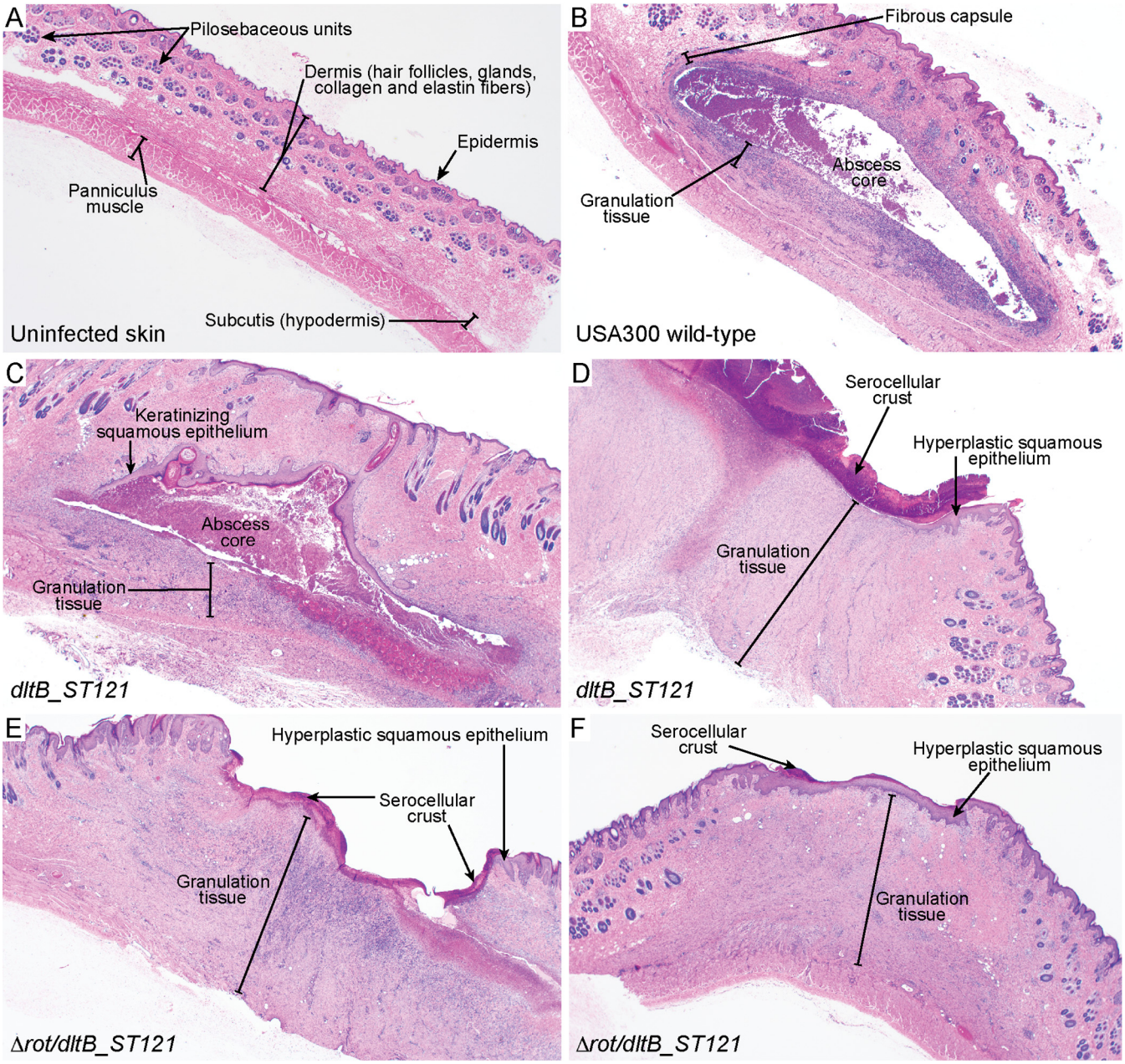

FIG 4 Histopathological analysis of rabbit skin lesion types caused by USA300 and *dltB*_ST121 and *dltB*_ST121/Δ*rot* isogenic mutants during reinfection. (A) Representative image of normal rabbit skin. (B) A dermal abscess with a necrotic center surrounded by organizing granulation tissue and inflammatory cells. (C) Keratinizing cyst that develops when restorative epithelium covering an ulcer becomes trapped in the dermis without communicating with the overlying epithelium. The cyst roof is keratinizing while the floor remains acutely inflamed, and the subjacent dermis and subcutis are replaced by organizing granulation tissue. (D) An ulcer covered by a thick serocellular crust. The epidermis is absent at the ulcer center, and the area deep to the ulcer is organized granulation tissue, which extends through the subcutis and panniculus muscle. (E) Skin ulcer. A serocellular crust overlies a bed of organizing granulation tissue. A cyst is developing in the tissue shown in the lower right corner of the image. (F) Resolving ulcer. A serocellular crust sits atop a layer of hyperplastic squamous epithelium that has reepithelialized an ulcer. An organizing bed of granulation tissue lies deep to the epithelium. Panels B to F represent skin pathology at 14 days after secondary infection. The original magnification is ×20.

experiments and had thus been previously exposed to *S. aureus*. Consistent with this idea, virtually all humans have anti-*S. aureus* antibodies in circulation, a result of current or prior exposure to *S. aureus* (colonization and/or infection).

Localized tissue innate immunity can also contribute to protection during recurrent infection, a notion supported by the work of Lam et al. (43). Studies first performed in the 1920s reported that nonspecific local cutaneous immunity to *S. aureus* infection in rabbits could be induced by bacterial broth filtrates or by primary infection (44, 45).

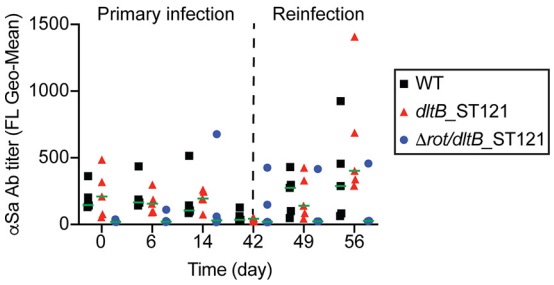

FIG 5 Reinfection does not significantly increase the level of anti-*S. aureus* antibodies in rabbit blood. The level of anti-*S. aureus* (αSa) antibodies (IgG) in serum of infected rabbits was assessed by flow cytometry as described in Materials and Methods. Each symbol represents a unique animal. Ab, antibody.

This response lasted for at least 5 to 6 weeks and was contained to a relatively narrow area around the primary injection site (44). Protective immunity against *S. aureus* SSTIs was elucidated further in a mouse model of recurrent infection by Chan et al. (46, 47). This research group determined that localized skin immune memory is time dependent and involves interleukin-17 (IL-17), gamma interferon (IFN-γ), and macrophage-conferred memory.

In summary, *rot* and/or *dltB* modifications can be engineered in important *S. aureus* strains such as USA300 and thereby reduce the inoculum required to develop reproducible skin disease in rabbits. Large inocula that are typical of current infection models likely overwhelm the host immune system and fail to mimic the natural course of infection. Reduction of the inoculum is a first step toward developing an *S. aureus* infection model that can better approximate human infection. Although we used the low-dose rabbit infection model to test recurrent *S. aureus* disease, this animal model can be extended readily to include testing of potential therapeutics and/or vaccine antigens.

MATERIALS AND METHODS

Ethics statement. All animal studies and procedures were reviewed and approved by the Animal Care and Use Committee at Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases (NIAID) (protocol numbers 2017-011E and 2018-037E). Work was carried out in accordance with the institutional guidelines for animal use and followed the guidelines and basic principles in the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, the Animal Welfare Act, and the Guide for the Care and Use of Laboratory Animals and conformed to the guidelines of the National Institutes of Health (NIH).

Bacterial strains and culture conditions. *S. aureus* strain LAC is a community-acquired USA300 isolate that has been characterized previously (48). Unless stated otherwise, *S. aureus* strains were grown in tryptic soy broth (TSB; Millipore-Sigma) at 37°C with constant shaking at 225 rpm. For preparation of the inoculum, overnight culture was diluted 1:200 into fresh medium and cultured to the early stationary phase of growth (optical density at 600 nm [OD_{600}] of ~2.0). Subsequently, bacteria were collected by centrifugation (4,000 $\times$ *g* for 10 min at 4°C), washed, and suspended in sterile, injection-grade saline at the desired concentration (10^3 to 10^8 CFU/injection). Each inoculum preparation was verified by plating 100 μL of serial 10-fold dilutions on tryptic soy agar (TSA) plates. CFU were enumerated after 24 h of growth at 37°C.

Generation of *dltB* variant alleles and *rot* deletion in USA300. Isogenic USA300 mutant strains with deletions of *rot* (Δ*rot*) and/or the indicated *dltB* alleles, ST1 (USA300Δ*rot/dltB*ST1), ST96 (USA300Δ*rot/dltB*ST96), or ST121 (USA300*dltB*ST121 and USA300Δ*rot/dltB*ST121), were generated by allelic replacement using vector pKOR1 as described previously (49). The SNPs engineered in the USA300 *dltB* gene (see Fig. S1 in the supplemental material) conferred the following amino acid substitutions in the USA300 DltB protein: I2T and *405Q in ST1; K402R and *405Q in ST96; and T113K, Y250H, and *405Y in ST121. Primers used for the replacement of *dltB* alleles and *rot* deletion are listed in Table S1. For the ST1 *dltB* allele, three PCR products (PCR1 generated with primer pair 16F-16R1, PCR2 generated with primer pair 16F2-16R2, and PCR3 with primer pair 16F3-16R) were stitched together and cloned in pKOR1. For the ST96 *dltB* allele, two PCR products (PCR4 amplified with primer pair 16F-16R3 and PCR5 amplified with primer pair 16F4-16R) were stitched together. For the ST121 *dltB* allele, four PCR products (PCR6 from primer pair 16F-16R4, PCR7 from primer pair 16F5-16R5, PCR8 from primer pair 16F6-16R6, and PCR9 from primer pair 16F7-16R) were stitched together. For the *rot* deletion, two PCR products flanking the *rot* gene alleles were generated using primers pairs 17F and 17R1, and 17F2 and 17R. The two PCR products were ligated into vector pKOR1 for allelic replacement on the chromosome

of strain USA300 LAC. *dltB* allele replacements were performed subsequently. Candidate strains carrying deletions of *rot* and allelic replacements of *dltB* were verified by PCR and DNA sequencing.

Rabbit SSTI model. Virulence of USA300 wild-type and isogenic mutant strains was evaluated in a rabbit skin and soft tissue infection model, as described previously (33). Each rabbit was inoculated subcutaneously in the left and right flank with 100 μL of sterile injection-grade saline containing the indicated bacterial inoculum (at each site). Animals were checked at least once per day after inoculation to determine health status and to evaluate skin disease. Sizes of abscesses or regions of inflammation were measured with calipers. One animal (2 sites) per inoculum/strain was used for the pilot experiment.

Five animals (10 abscesses) per *S. aureus* strain were used to model recurrent SSTI. *S. aureus* abscesses (10^6 CFU/injection site) were allowed to resolve until no palpable abscess was present. Animals were then allowed to recover for an additional week. Subsequently, the site of infection was reshaved, treated with Nair, and cleansed with 70% ethanol. Animals were reinfected with 10^8 CFU of *S. aureus* by subcutaneous inoculation. The locations of primary and secondary inoculations were similar but not identical. Animals were checked at least once daily for 14 days after inoculation to determine health and abscess development. Abscess size and dimensions were recorded each day. On day 14, animals were euthanized and areas of skin containing lesions were collected for histopathology analyses.

Histopathology. Tissue specimens were fixed by immersion in 10% neutral buffered formalin for a minimum of 7 days. Tissue samples were processed with a Sakura VIP-6 Tissue Tek on a 12-h automated schedule using a graded series of ethanol, xylene, and paraffin. Embedded tissues were sectioned at approximately 5 μm, dried overnight at 42°C, and stained with hematoxylin and eosin (HE) for histological examination by a board-certified veterinary pathologist. Images were adjusted for contrast and brightness using Adobe Photoshop version 22.5.0.

Determination of anti-*S. aureus* antibody titer in rabbit blood. To determine the level of anti-*S. aureus* antibody in rabbit serum, we used flow cytometry to measure antibody deposition on the surface of intact bacteria. Rabbit blood was collected into BD Vacutainer SST collection tubes according to the schedule outlined in Fig. 2. Samples were incubated at room temperature for approximately 20 to 30 min, and serum was separated by centrifugation at 1,100 $\times$ *g* for 10 min. To prevent nonspecific binding of rabbit antibody Fc regions to protein A on the surface of *S. aureus*, serum samples were incubated (blocked) with *S. aureus* protein A at 10 μg/mL (Calbiochem/EMD Millipore Corp.) for 3 h at 4°C. Rabbit polyclonal antiserum specific for *S. aureus* was used as a positive control (50).

Bacteria were cultured to mid-logarithmic phase of growth (OD_{600} = 0.75) in TSB as described above. Next, bacteria were pelleted by centrifugation (2,400 $\times$ *g* for 4 min at room temperature), washed once with phosphate-buffered saline (PBS), and suspended in blocking buffer (2% bovine serum albumin [BSA] in Dulbecco's PBS [DPBS]). Samples were blocked on ice for 1 h, centrifuged, and suspended in DPBS. One hundred microliters of bacterial suspension was mixed with blocked serum from the rabbit infected with the corresponding bacterial strain (final dilution of serum = 1:2,000) and incubated on ice for 30 min. Subsequently, bacteria were washed with 0.8% BSA in DPBS (wash buffer) followed by 30 min of incubation on ice with secondary antibody [fluorescein isothiocyanate (FITC)-conjugated goat F $(ab')_2$ fragment antibody specific for rabbit IgG(H+L); Jackson ImmunoResearch, West Grove, PA] used at a 1:500 dilution in DPBS. To remove unbound antibody, bacteria were washed again and suspended in wash buffer. Samples were analyzed by flow cytometry (FACSCelesta flow cytometer; BD Biosciences), and 50,000 events were collected per sample.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 9.1.1 (GraphPad Software LLC, San Diego, CA). Data were evaluated with a one-way analysis of variance (ANOVA) and Tukey's posttest to correct for multiple comparisons.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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We declare no conflicts of interest.

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