# On the Role of *Staphylococcus aureus* Sortase and Sortase-Catalyzed Surface Protein Anchoring in Murine Septic Arthritis

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**Anchoring of *Staphylococcus aureus* surface protein to the cell wall is catalyzed by sortase, a transpeptidase. The contribution of staphylococcal surface proteins to establishment of infection was examined using a murine septic arthritis model. Intravenous inoculation of mice with the sortase-deficient mutant *S. aureus* strain SMK3 did not result in weight loss or severe septic arthritis, in contrast to the parent strain, *S. aureus* Newman. Direct inoculation of the sortase mutant into joint cavities also failed to cause severe synovitis or erosive arthritis. Furthermore, intravenous inoculation with staphylococci resulted in the rapid clearing of the sortase mutant from the bloodstream. This phenomenon demonstrates the involvement of host neutrophils; when these cells were depleted, sortase mutant staphylococci caused severe systemic infection, although not septic arthritis. These results suggest that sortase mutant staphylococci are significantly less virulent than the parent strain, Newman: the sortase mutant has decreased ability to reach target organs and, once there, to induce an inflammatory response.**

*Staphylococcus aureus* infections in humans are associated with high morbidity and mortality. The emergence of *S. aureus* strains that are resistant to multiple drugs presents a therapeutic challenge to the use of conventional antibiotics and warrants a search for novel targets of anti-infective therapy. *S. aureus* expresses a repertoire of virulence factors, including exotoxins, exoenzymes, and adhesins, that contribute to the pathogenesis of disease [1]. Adhesins, such as fibronectin-, fibrinogen-, vitronectin-, and collagen-binding proteins, are surface proteins that mediate adherence to the extracellular matrix, which is the first critical event in the pathogenesis of most infections [2, 3]. During septic arthritis and osteomyelitis, collagen adhesin and bone sialoprotein are believed to act as primary virulence factors [4–6]. Furthermore, fibrinogen-binding proteins mediate adherence to multiple tissues and play a major role in the pathogenesis of septic arthritis, endocarditis, and other infectious diseases [7–9].

Staphylococcal adhesins are linked to the cell wall by sortase, an enzyme that cleaves polypeptides at a conserved LPXTG motif [10–12]. Cell-wall anchoring of surface proteins requires a C-terminal sorting signal that, in addition to an LPXTG motif, contains a C-terminal hydrophobic domain and a tail of mostly positively charged residues [13, 14]. Sorting signals have been identified in 20 surface proteins of *S. aureus* [15]. Mutants that lack the *srtA* gene appear to be defective in anchoring and display of many, if not all, surface proteins that bear sorting signals with an LPXTG motif [16]. Similar sorting signals have been found in the surface proteins of other gram-positive bacteria, including streptococci, clostridia, corynebacteria, lactococci, enterococci, and *Listeria* [3, 17, 18].

The role of surface proteins in the pathogenesis of bacterial infections has been studied using *S. aureus* variants that lack the *srtA* gene [16]. When an acute disease model is used, sortase mutants display a 1.5-log reduction in virulence, compared with the wild-type Newman strain, and a 2–3-log reduction in virulence after intravenous inoculation of bacteria, when the ability of staphylococci to establish kidney infections is measured [16]. Knockout mutations of the *srtA* gene in *Streptococcus gordonii* also interfere with cell-wall anchoring and display of surface proteins, accompanied by a loss of bacterial adhesive properties [19]. Previous work has left unresolved the question of whether the sortase-catalyzed transpeptidation reaction is involved in the pathogenesis of staphylococcal infections other than acute disease. The aim of the present study was to assess whether sortase-catalyzed anchoring of surface proteins is required for the establishment of septic arthritis and to ascertain whether sortase could be a promising future target for therapy.

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## Materials and Methods

*Mice.* Female NMRI and BALB/c mice, 6–8 weeks old, were purchased from B&K Universal AB and maintained in the animal facility of the Department of Rheumatology, University of Göteborg (Göteborg, Sweden). Mice were housed under standard conditions of light and temperature and were fed standard laboratory chow and water ad libitum.

*Bacterial strains.* The present study used the human clinical isolate *S. aureus* Newman (wild type) [20] and its isogenic sortase-deficient variant, strain SKM3 [16]. SKM3, which carries the wild-type *srtA* gene on a plasmid (pSrtA), was used as a control for *trans*-complementation [16]. This complemented strain was only used in the first experiment, because the plasmid did not show 100% stability (stability was 55%–97%). Before inoculation of animals, staphylococci were cultured for 24 h on tryptic soy agar (TSA), reinoculated, and grown on TSA for another 24 h. Staphylococci were then harvested and stored frozen at −20°C, after aliquots had been suspended in PBS supplemented with 5% bovine serum albumin and 10% dimethyl sulfoxide. Before inoculation into mice, staphylococcal suspensions were thawed, and the bacteria were washed in PBS and then diluted in PBS to achieve the appropriate staphylococcal inoculum size. Inocula were injected intravenously into the tail (0.2 mL of staphylococcal suspension) or directly into the knee cavity (0.02 mL) of mice. To determine the number of colony-forming units that were injected into animals, aliquots of staphylococcal suspensions were diluted and plated on TSA, and colonies were counted.

*Animal infections.* Four independent in vivo animal experiments and 1 in vitro experiment were performed. NMRI mice were used in the first 4 experiments and BALB/c mice in the fifth. First, to evaluate the effect of sortase on the development of hematogenous septic arthritis, 3 groups of mice (10 animals in each group) were inoculated with $6 \times 10^6$ cfu/mouse of *S. aureus* Newman, the sortase mutant, or the complemented mutant strain. Mice were regularly weighed and examined for arthritis until death or until they were killed (at day 14). Second, to investigate the effect of sortase expression on the kinetics of *S. aureus,* 15 mice were inoculated with $6 \times 10^6$ cfu/mouse of *S. aureus* Newman, and another 15 mice were inoculated with the sortase-deficient mutant strain SKM3. Forty-eight hours after the inoculations, 7 mice in each group were killed, and culture samples were obtained from blood, kidneys, and joints. Blood samples were also obtained from these mice 24 h after inoculation. The remaining mice in each group (7 mice that were inoculated with the Newman strain and 8 mice that were inoculated with the SKM3 strain) were subjected to bleeding procedure for bacterial culturing at day 3 and then killed at day 7 after inoculation, at which point samples were obtained from kidneys and joints. Serum from these mice was used to determine interleukin (IL)–6 levels, a marker for the inflammatory response to staphylococcal infection. During the course of this experiment, 1 mouse that had been injected with *S. aureus* Newman died. Third, 6 mice were inoculated intra-articularly in the left knee joint with $2.4 \times 10^4$ cfu/mouse of *S. aureus* Newman, and another 6 mice were inoculated with $2.9 \times 10^4$ cfu/mouse of the SKM3 strain. The contralateral joint was used as the control and was injected with the same volume of PBS. Animals were killed 3 days after inoculation, and the joints were examined histologically for signs of inflammation. Fourth, an in vitro phagocytosis experiment was done to evaluate the role of surface proteins in elimination of staphylococci. Fifth, to evaluate the role of neutrophils in the clearance of sortase mutant bacteria, 8 granulocyte-depleted BALB/c mice and 8 control mice were inoculated with the sortase mutant strain. Infection was clinically evaluated for 1 week, and kidneys were analyzed for bacteria load.

*Clinical evaluation of arthritis.* All mice were observed intermittently during the course of these experiments, and limbs were inspected at regular intervals by a single observer (I.-M.J.) who was blinded to the clinical parameters for the treatment groups. To evaluate the intensity of arthritis, a clinical scoring system of 0–3 points for each paw was used (1, mild visible swelling and/or erythema; 2, moderate swelling and erythema; and 3, marked swelling and erythema). The arthritis index was constructed by adding the scores from all 4 limbs for each animal.

*Bacteriologic examination of infected animals.* Mice that were inoculated intravenously with $6 \times 10^6$ cfu of *S. aureus* were killed 48 h or 7 days after inoculation. Culture samples from blood were obtained 24 h and 48 h or 3 days and 7 days after bacterial inoculation. The talocrural and radiocarpal joints were dissected aseptically, and culture samples were obtained with cotton sticks and then cultured on TSA plates containing 5% horse blood for 24 h. All bacteria loads in the joints that were $> 10^4$ were set to $10^4$. The kidneys were aseptically removed at the time of death, homogenized, and diluted to appropriate concentrations in PBS. One hundred microliters of the homogenate was transferred to blood agar plates and cultured for 24 h, and the colony-forming units were counted. Bacteria colonies were tested for catalase and coagulase activity.

*Analysis of IL-6.* Serum samples from mice participating in the second in vivo experiment were used to analyze IL-6. The murine hybridoma cell line B9 is dependent on IL-6 for growth and was used as an indicator to determine the serum levels of IL-6 [21, 22]. The B9 cells were seeded into microtiter plates (5000 cells/well), and dilutions of the serum samples were added to each well. After 68 h of incubation, [$^3$H]thymidine (Radiochemical Centre) was added, and the cells were harvested 6 h later. IL-6–dependent cell proliferation was measured as the incorporation of [$^3$H]thymidine by use of liquid scintillation counting and compared with a reference sample that had been treated with purified recombinant IL-6. Previous work has shown that the growth of B9 cells is not stimulated by the addition of several recombinant cytokines, including IL-1$\alpha$, IL-1$\beta$, IL-2, IL-3, IL-5, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor–$\alpha$, and interferon-$\gamma$. Only a weak stimulation of cell growth was observed when B9 cells were treated with IL-4 [22].

*Histopathologic examination.* The knee joints from mice injected intra-articularly with staphylococci were collected at day 3 after the inoculation. Histopathologic examination was performed after routine paraformaldehyde fixation, decalcification, and paraffin embedding. Tissue sections were stained with hematoxylin and eosin. The joints were studied by a single blinded examiner (I.-M.J.). Synovial hypertrophy was defined as a synovial membrane thickness of $> 2$ cell layers [21]. Histologic scoring was used to quantitate the degree of synovial hypertrophy and degradation of cartilage and/or bone. Scores were set at 1 point for mild, 2 points for moderate, and 3 points for severe synovitis and erosive joint damage [23].

*Phagocytosis and intracellular killing of bacteria.* Replicate samples from 4 mice were used for a phagocytosis and intracellular killing assay, using the method of Lissner et al. [24]. In brief, peritoneal macrophages were collected by injection of 3 mL of ice-cold medium (Iscove's complete medium that contained 100 $\mu$g/mL of gentamicin) into the peritoneal cavity of NMRI mice. After a 1-min massage of the abdomen, the medium was aspirated, and macrophages were adjusted to a concentration of $2 \times 10^6$ cells/mL. The cells were seeded in a volume of 200 $\mu$L into 24-well plates (Nunc) and incubated at room temperature for 90 min. Five hundred microliters of medium was added to each well, and the cells were incubated for 4 h at 37°C. The medium then was removed and was replaced by 500 $\mu$L of a new, antibiotic-free medium. After incubation overnight at 37°C, the cells were washed in Iscove's medium and 500 $\mu$L of a suspension with $2 \times 10^6$ cfu/mL of the wild-type *S. aureus* Newman strain or the sortase-deficient mutant. After 50 min of incubation, the cells were washed 3 times to remove nonphagocytosed bacteria. Macrophages were analyzed for intracellular bacteria content at 3 different time periods, beginning immediately after incubation with bacteria and 4 and 24 h later. Gentamicin was added to cell cultures, and the cultures were incubated for 4 or 24 h to avoid extracellular replication of bacteria. After the cells were washed in Iscove's medium, the macrophages were lysed by exposure to distilled water for 20 min. The lysate was diluted 1:1, 1:10, 1:100, and 1:1000 and plated on 5% horse blood agar plates, and colony-forming units were counted after overnight incubation.

*Neutrophil depletion.* Mice were depleted of granulocytes by intraperitoneal injection of RB6-8C5 antibody, a rat IgG2b that selectively depletes mature mouse neutrophils [25]. Eight BALB/c mice received 1 mg of the RB6-8C5 antibody, and 8 mice received the corresponding amount of an IgG rat anti-ovalbumin control antibody. Two hours later, the mice were inoculated with $7 \times 10^6$ cfu of sortase mutant *S. aureus.* The course of infection was observed for 1 week. After the mice were killed, the kidneys were analyzed for bacteria load.

*Statistical analyses.* Statistical evaluations were made using the Mann-Whitney *U* test or Fisher's exact test. All values are reported as median and interquartile range (IQR). Survival data were analyzed with the Kaplan-Meier log-rank test.

## Results

*Role of staphylococcal sortase during the clinical course of infection.* Mice infected with the wild-type *S. aureus* Newman strain died from intravenous infections at day 7 (mortality rate, 30%), 10 (mortality rate, 60%), or 14 (mortality rate, 80%) (figure 1). No lethal infections were observed for mice infected with the sortase mutant strain SKM3 ($P < .01$) (figure 1). Mice infected with *S. aureus* Newman or the SKM3 (pSrtA) strain had dramatic weight loss, whereas the body weight of mice infected with the sortase mutant strain SKM3 remained largely unaffected (figure 2). *S. aureus* Newman, the human clinical isolate and wild-type strain used in our experiments, caused infectious arthritis in 50%, 89%, and 100% of infected animals (10 mice/group) at days 3, 7, and 14 after inoculation, respectively. In contrast, the sortase mutant strain SKM3 caused infectious arthritis in 30% (day 3) and 10% (days 7 and 14, respectively; $P < .01$ and $P < .05$, compared with mice that received the Newman strain) of the experimental animals. The SKM3 (pSrtA) strain caused an infectious arthritis in 80% (day 3) and 100% (days 7 and 14) of all infected animals, which demonstrated that complementation of the sorting defect restored staphylococcal virulence.

The median arthritis index score (a measure of the severity of infectious arthritis) for mice infected with *S. aureus* Newman increased from 0.5 (IQR, 0–2.0) on day 3 to 2.0 (IQR, 1.0–4.3) and 2.5 (IQR, 2.0–3.0) on days 7 and 14, respectively. The sortase mutant strain SKM3 produced median arthritis index scores of 0 (IQR, 0–1.0) and 0 (IQR, 0–0) during the same time period (figure 3). Complementation of the sortase mutant phenotype restored pathogenicity, and median arthritis index scores of 1.5 (IQR, 1.0–2.0), 3.0 (IQR, 1.0–4.0), and 4.0 (IQR, 2.0–6.0) were seen on days 3, 7, and 14, respectively. Thus, sortase is important in the establishment of staphylococcal infectious arthritis. These findings are consistent with the view that staphylococci require adhesion of surface protein to bone and cartilage tissues to establish a productive infection.

*Trans*-complementation with plasmid-encoded sortase resulted in weight loss during infection in mice, but this did not indicate that the virulence defect that prevented strain SKM3 from causing an acute lethal infection had been corrected; only 10% of the SKM3 (pSrtA)–infected animals died (figure 1). These data suggest that the regulation of sortase expression or a gene-dosage effect of sortase may play an important role during infection.

*Staphylococcal persistence in host tissues.* We wondered whether sortase and sortase-anchored surface proteins are required for staphylococcal multiplication and survival during infection. Fifteen mice were inoculated with $6 \times 10^6$ cfu/mouse of *S. aureus* Newman (wild type), and 15 mice were inoculated with the sortase-deficient mutant strain SKM3. During the course

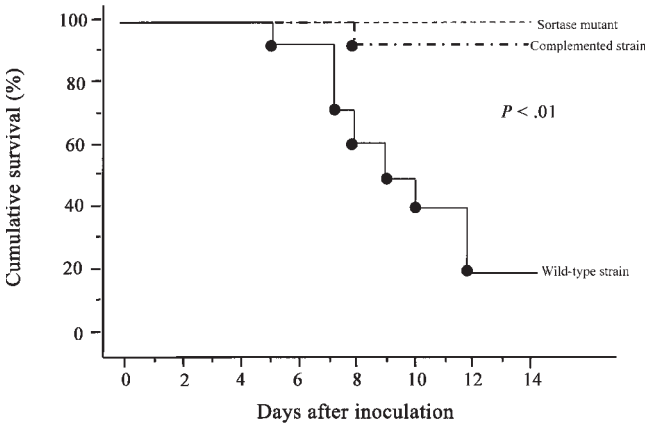


**Figure 1.** Cumulative survival of NMRI mice inoculated with $6 \times 10^6$ cfu of *Staphylococcus aureus* Newman; its isogenic sortase-deficient mutant, strain SKM3; or the sortase mutant complemented with the wild-type sortase gene on a plasmid (10 mice/group). For mice inoculated with *S. aureus* Newman vs. mice inoculated with the sortase mutant strain SKM3, $P < .01$, by the Kaplan-Meier log-rank test.

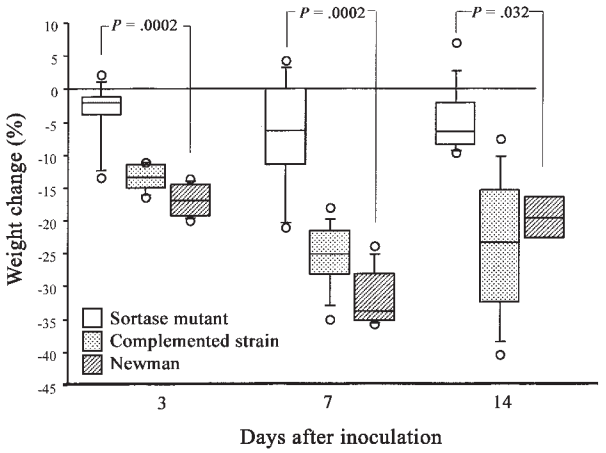


**Figure 2.** Changes in body weight in NMRI mice inoculated with $6 \times 10^6$ cfu of *Staphylococcus aureus* Newman; its isogenic sortase-deficient mutant, strain SKM3; or the sortase mutant complemented with the wild-type sortase gene on a plasmid (10 mice/group). Data shown are median and interquartile range for each group of mice. *P* values are for the comparison between the wild-type strain Newman and the sortase mutant strain SKM3.

of this experiment, 1 mouse that had been injected with *S. aureus* Newman died. Figure 4*A* shows staphylococcal persistence in blood. One day after injection of $6 \times 10^6$ cfu of *S. aureus* Newman, a median level of 770 cfu/mL of blood (IQR, 227–1563 cfu/mL) was found. Wild-type staphylococci persisted in the blood for 7 days; median levels of 270 cfu/mL (IQR, 170–570 cfu/mL), 20 cfu/mL (IQR, 5–60 cfu/mL), and 63 cfu/mL (IQR, 0–144 cfu/mL) were isolated on days 2, 3, and 7, respectively. In contrast, the sortase mutant strain SKM3 was almost immediately cleared from the bloodstream and could be isolated only in very small amounts from the blood at any time during infection (figure 4*A*).

On day 2 after bacterial inoculation, the joints of only 1 of 7 mice infected with the sortase-deficient mutant had bacterial growth. In contrast, all of the mice inoculated with wild-type staphylococci displayed bacterial growth in joints (range, 5 to $\geqslant 10^4$ cfu). On day 7 after bacterial inoculation, 4 of 8 mice inoculated with the sortase mutant had bacterial growth in joints, whereas, again, all of the mice inoculated with wild-type staphylococci showed bacterial growth in joints. In the latter group, the number of bacteria was very high in many joints (figure 4*B*).

A similar result was obtained when we analyzed staphylococcal persistence in kidney tissues. *S. aureus* Newman replicated to very high numbers during infection: median levels of $7.1 \times 10^7$cfu (IQR, $1.3 \times 10^7$ to $8.5 \times 10^7$ cfu) and $4.5 \times 10^7$ cfu (IQR, $0.8 \times 10^7$ to $7.6 \times 10^7$ cfu) were found in kidney tissues on days 2 and 7, respectively. Growth of *S. aureus* SKM3 was reduced: median levels of $4.6 \times 10^4$ cfu (IQR, $1.0 \times 10^4$ to $11.3 \times 10^4$ cfu) and $4.0 \times 10^6$ cfu (IQR, $0.3 \times 10^6$ to $6.3 \times 10^6$ cfu) were isolated from the kidneys at the same time points (figure 4*C*). These results reveal that sortase and sortase-anchored surface proteins are required for staphylococcal persistence in the host.

*Stimulation of the immune response during staphylococcal infection.* Serum levels of IL-6 were significantly lower in mice that were infected with the sortase mutant strain than in mice inoculated with *S. aureus* Newman. On day 2 after inoculation, median IL-6 levels were 950 pg/mL (IQR, 360–1600 pg/mL) for the sortase mutant strain and 12,800 pg/mL (IQR, 2200–19,000 pg/mL) for the wild-type strain (figure 5). A large difference in IL-6 concentrations was also observed on day 7 after inoculation. These results suggest that infections with wild-type staphylococci elicit a much more profound inflammatory response than do infections with the sortase mutant strain.

*Direct arthritogenicity of sortase-expressing staphylococci.* We wondered whether the diminished arthritogenicity of the sortase mutant strain is due to the increased elimination of staphylococci before they can infect the joint or whether it is caused by the decreased virulence of staphylococci in situ. To distinguish between these possibilities, staphylococci were administered directly into the articular cavity in mice, thereby bypassing the requirement that intravenously injected staphylococci spread systemically to cause arthritis. Five of 7 mice that were infected with the wild-type strain Newman showed moderate to severe synovitis (histologic score $\geqslant 2$), whereas none of the mice infected with the sortase mutant strain displayed signs of severe inflammation ($P = .02$). Indeed, the latter group showed only a mild synovitis (histologic score $\leqslant 1$). Moreover, 4 of the mice injected with the wild-type strain had erosion of cartilage and/or bone in the knee joints, whereas none of the mice injected intra-articularly with sortase mutant showed signs

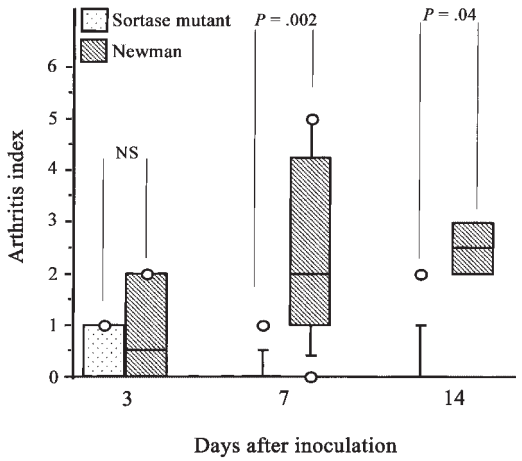


**Figure 3.** Severity of arthritis in NMRI mice inoculated with $6 \times 10^6$ cfu of *Staphylococcus aureus* Newman or its isogenic sortase-deficient mutant, strain SKM3 (10 mice/group). Data shown are median and interquartile range for each group of mice. Comparisons were made using the Mann-Whitney *U* test. *P* values are for the comparison between the wild-type Newman strain and the sortase mutant strain SKM3. NS, not significant.

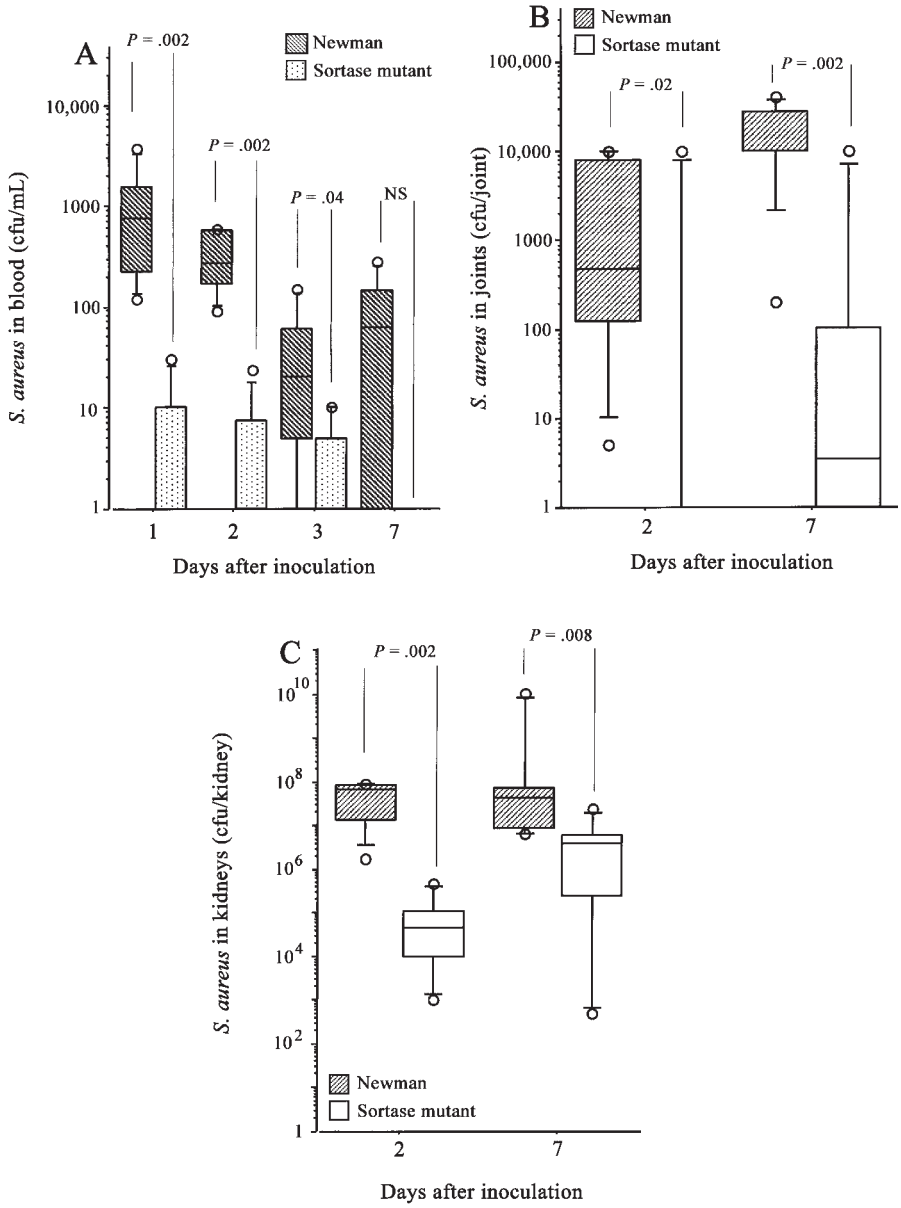


**Figure 4.** Persistence of *Staphylococcus aureus* in blood *(A),* early homing of staphylococci to the joints *(B),* and bacteria load in the kidneys *(C)* of NMRI mice inoculated with $6 \times 10^6$ cfu of *S. aureus* Newman or its isogenic sortase-deficient mutant, strain SKM3 (15 mice/group). Blood samples were obtained for culture at days 1, 2, 3, and 7 from mice inoculated with the Newman strain (7 mice/time point) and from mice inoculated with the sortase mutant strain SKM3 (7 or 8 mice/time point). Samples from joints and kidneys were taken after mice were killed, on days 2 and 7. Data are median and interquartile range for each group of mice. Comparisons were made using the Mann-Whitney *U* test. NS, not significant.

of cartilage or bone erosion (*P* was not significant) (figure 6). The joints of control mice all had negative results of examination for inflammation (data not shown).

*Phagocytosis and intracellular killing of sortase mutant staphylococci.* Because *S. aureus* SKM3 cannot display surface proteins, we wondered whether sortase mutant staphylococci were more rapidly phagocytosed and killed than wild-type staphylococci. To our surprise, no difference in macrophage phagocytosis or killing could be detected between *S. aureus* Newman and the sortase mutant strain SKM3 (figure 7).

*Effect of neutrophil depletion on infection induced by sortase mutant* S. aureus. Polymorphonuclear neutrophils (PMNs) are phagocytic cells that represent an important component of the innate immune response against staphylococcal infection. We presumed that PMNs play a role in eliminating sortase mutant staphylococci during infection. Indeed, kidneys from mice de-

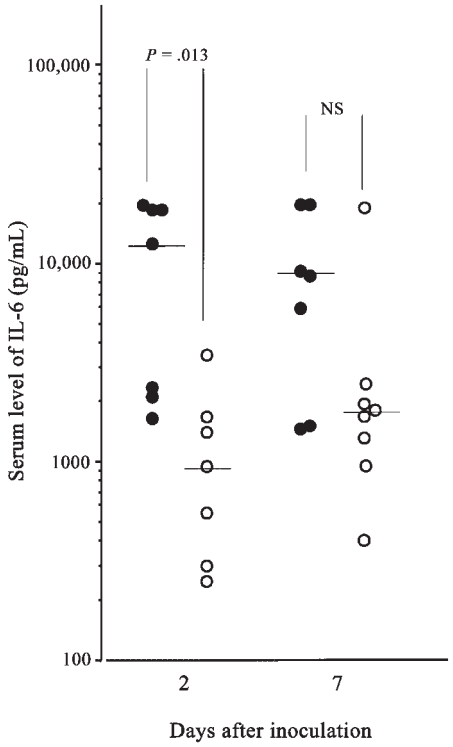


**Figure 5.** Serum levels of interleukin (IL)–6 after inoculation with $6 \times 10^6$ cfu of *Staphylococcus aureus* Newman *(filled circles)* or its isogenic sortase-deficient mutant, strain SKM3 *(open circles)* (7 mice/group at day 2 and 7 or 8 mice/group at day 7). Comparisons were made using the Mann-Whitney *U* test. Horizontal bars represent median values. NS, not significant.

pleted of PMNs harbored significantly more staphylococci than kidneys from mice that received the control antibody (median level, $12.5 \times 10^7$ cfu [IQR, $0.1 \times 10^7$ to $19.3 \times 10^7$ cfu] vs. $1.8 \times 10^5$ cfu [IQR, $0.6 \times 10^5$ to $17.5 \times 10^5$ cfu]; $P = .04$). One of the neutrophil-depleted mice died within 1 week of inoculation. The neutrophil-depleted mice also lost significantly more weight than did the control mice. On day 3 after staphylococcal inoculation, the median weight decrease was 16.0% (IQR, 11.5%–21.6%) for neutrophil-depleted mice, whereas nondepleted control mice lost only 0.2% of their original weight, with an IQR of −0.9% to 3.3% ($P < .01$). One week after the inoculation, the median weight decrease among neutrophil-depleted mice was even more pronounced, at 38.8% (IQR, 3.3%–40.9%), whereas nondepleted control mice had a median weight increase of 0.2% (IQR for weight change, −4.9% to 3.8%) ($P < .05$). Only 1 mouse in each group displayed mild arthritis during the course of the experiment.

## Discussion

The *srtA* gene product has been shown to be necessary for the cell-wall anchoring of many surface proteins, and a deletion of the *srtA* gene abrogates the display of surface proteins by mutant *S. aureus* strains [16]. Because several adhesins, including protein A, clumping factor, fibronectin-binding proteins, collagen adhesin, and bone sialoprotein, are considered to be virulence factors [4, 5, 8, 9, 26, 27], we wondered whether the absence of all these molecules as a result of inactivation of the sortase gene would have an impact on staphylococcal virulence in a murine model of septic arthritis. We report here that mice inoculated with the sortase mutant had a significantly higher rate of survival than mice inoculated with the wild-type parent strain. Mice infected with the sortase mutant failed to develop significant histopathologic signs of arthritis. Furthermore, mice that were inoculated with the sortase mutant did not show signs of severe infection, as indicated by the absence of a weight decrease and by decreased production of inflammatory cytokines, compared with mice that had been inoculated with the isogenic wild-type parent strain. Thus, sortase and the sortase-catalyzed anchoring of surface proteins promote the establishment of murine septic arthritis and are prerequisites to the development of severe disease after intravenous injection.

The phenotypic defects of sortase mutants can be explained in 1 of 2 ways: (1) staphylococci that lack surface proteins ("naked staphylococci") may be more rapidly phagocytosed and killed than are wild-type *S. aureus* strains, or (2) sortase mutants may be defective in adhering to organ tissues and therefore cannot colonize target tissues such as bone or cartilage. In an effort to distinguish between these possibilities, we performed several experiments. First, after intravenous injection of staphylococci, the sortase mutant strain was much more rapidly cleared

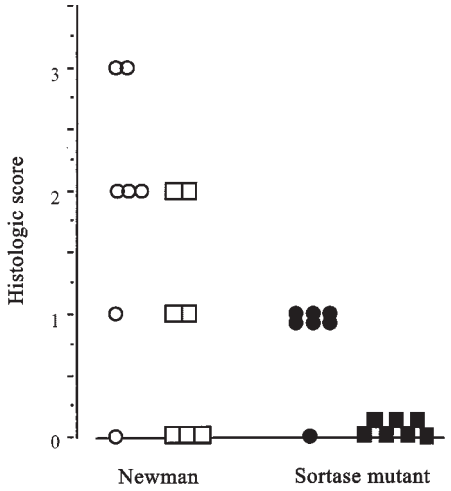


**Figure 6.** Histopathologic evaluation of knee joints from NMRI mice 3 days after intra-articular injection with $2.4 \times 10^4$ cfu of *Staphylococcus aureus* Newman or $2.9 \times 10^4$ cfu of the isogenic sortase-deficient mutant strain SKM3 (7 mice/group). Histopathologic scores were used to assess the degree of synovial hypertrophy *(circles)* and erosive degradation of cartilage and/or bone *(squares)*. Scores were set at 1 point for mild, 2 points for moderate, and 3 points for severe synovitis and erosive joint damage.

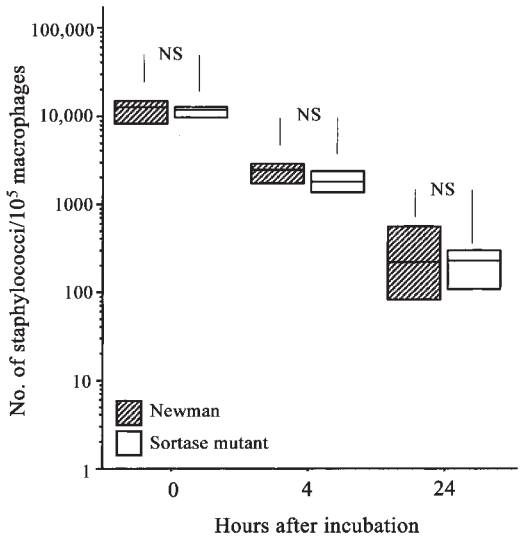


**Figure 7.** Intracellular content of bacteria at the time of ingestion (0 h) and after intracellular killing 4 and 24 h after the incubation of peritoneal macrophages with the parental *Staphylococcus aureus* Newman or the sortase-deficient mutant SKM3. Comparisons were made using the Mann-Whitney *U* test. NS, not significant.

from the bloodstream than was the wild-type parent strain. Second, in in vitro phagocytosis experiments used to test whether the disappearance of staphylococci from blood was caused by more-rapid phagocytosis, we could detect no differences in the rate of uptake or killing of the wild-type and the sortase mutant strains by peritoneal macrophages. Although these experiments suggest that macrophages may not be responsible for the rapid clearing of the sortase mutant strain from the blood stream, it is also conceivable that macrophage killing requires blood factors that were absent from our assay. Further work is needed to address this question. Third, intra-articular injection of sortase mutant staphylococci into joints failed to produce severe arthritis. Even if the sortase mutant strain was not cleared from the bloodstream and peripheral tissues, these "naked staphylococci" cannot adhere to target tissues, and they display severe defects in establishing localized infections. In summary, we think it is likely that surface proteins of staphylococci contribute to both the survival (resistance to phagocytic killing) of bacteria in blood and the adherence of bacteria to organ tissues, mechanisms that promote localized and generalized *S. aureus* infections.

Neutrophils play an important role as effector cells in the innate host defense against bacteria and are rapidly recruited to the site of infection [25]. To analyze the role of PMNs in clearing sortase mutant staphylococci from the bloodstream, we depleted this leukocyte population of neutrophils before inoculation of bacteria. Neutrophil-depleted mice experienced severe systemic infection after inoculation with the sortase mutant, as indicated by a dramatic weight loss. In spite of the severe systemic infection, which presumably was generated by the relatively unhindered multiplication of sortase mutant staphylococci in blood, the infected animals did not display signs of infectious arthritis. Thus, the ability of staphylococci to cause arthritis not only is the result of the survival of bacteria in the tissues but also requires very specific adherence mechanisms—for example, adherence mediated by collagen adhesin [4] and clumping factor [28].

In summary, we report that the sortase mutant *S. aureus* strain SMK3 is significantly less virulent than its parental strain, *S. aureus* Newman. The less severe outcome of infection is due to the decreased ability of the sortase mutant to reach target organs and to induce inflammatory response, combined with a more efficient elimination of the mutant strain by the host innate immune system.

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