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Original article

The role of *Staphylococcus aureus* sortase A and sortase B in murine arthritis

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Abstract

Gram-positive pathogenic bacteria display proteins on their surface that play important roles during infection. In *Staphylococcus aureus*, these surface proteins are anchored to the cell wall by two sortase enzymes, SrtA and SrtB, that recognize specific surface protein sorting signals. The role of sortase enzymes in bacterial virulence was examined using a murine septic arthritis model. Intravenous inoculation with any of the Δ(*srtA*), Δ(*srtB*) or Δ(*srtAB*) mutants resulted in significantly increased survival and significantly lower weight loss compared with the parental strain. Mice inoculated with the Δ(*srtA*) mutant did not express severe arthritis, while arthritis in mice inoculated with the Δ(*srtB*) mutant was not different from that seen in mice that were infected with the wild-type parent strain. Furthermore, persistence of staphylococci in kidneys and joints following intravenous inoculation of mice was more pronounced for wild-type and Δ(*srtB*) mutant strains than for Δ(*srtA*) or Δ(*srtAB*) variants. Together these results indicate that sortase B (*srtB*) plays a contributing role during the pathogenesis of staphylococcal infections, whereas sortase A (*srtA*) is an essential virulence factor for the establishment of septic arthritis.

Keywords: *Staphylococcus aureus*; Sortase; Adhesin; Septic arthris

1. Introduction

Staphylococcus aureus infections in humans are associated with high morbidity and mortality [1]. *S. aureus* produces a number of virulence factors, including exotoxins, exoenzymes, and adhesins that all contribute to the pathogenesis of disease [2,3]. The first critical event in infection is adherence of the staphylococci to the extracellular matrix, an event that is mediated by surface protein adhesins, e.g. collagen-adhesin bone sialoprotein or clumping factor among others [4]. Surface proteins are believed to be virulence factors in septic arthritis and osteomyelitis, as isogenic variants of *S. aureus* strains lacking specific surface protein genes are defective in the ability to establish these infections [5–8]. The genome of *S. aureus* harbors about twenty different genes that encode surface proteins with a C-terminal cell wall sorting signal and LPXTG motif [9,10]. The transpeptidase sortase A (SrtA) [11] links the surface protein adhesins to the cell wall by cleaving the polypeptides at the LPXTG motif, followed by covalent amide linkage of the C-terminal carboxyl group to cell wall crossbridges [12–15].

An essential co-factor for bacterial growth and other biological processes is the supply of molecular iron, and pathogenic bacteria have evolved processes for the acquisition of iron from their environment [16,17]. During animal infection, staphylococci scavenge heme-bound iron and presumably transport this molecule into the bacterial cytoplasm [18,19]. The staphylococcal *isd* locus encodes anchored surface proteins (IsdA, IsdB and IsdC), sortase B (SrtB), as well as lipoprotein ATPase and membrane permease, which are involved in the transport of heme-iron across the bacterial envelope [19]. In contrast to sortase A, which appears to be constitutively expressed, the *isd* locus is regulated by Fur, the ferric uptake repressor [19,20]. When iron concentrations are low, Fur permits transcription of the *isd* locus and cell wall anchoring of IsdC, the NPQTN motif-containing surface protein substrate of sortase B [19]. Although this has not yet been established in detail, it is conceivable that the cell wall anchored surface proteins encoded in the *isd* locus facilitate

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bacterial transport of heme-iron across the cell wall envelope, as both SrtA and SrtB are required for the transport of heme-iron into the bacterial cell [18].

Our earlier work demonstrated that sortase A, a transpeptidase responsible for anchoring of surface proteins with a LPXTG motif, plays an essential role in the pathogenesis of murine septic arthritis [21]. The aim of this study was to assess whether sortase B acts as a virulence factor and contributes to the outcome of septic arthritis, as well as to compare the phenotype of sortase A versus sortase B mutations in a model of murine septic arthritis.

2. Materials and methods

2.1. Mice

Female NMRI mice, 7 weeks old, were purchased from B&K universal AB (Sollentuna, Sweden) and maintained in the animal facility of the University of Göteborg.

2.2. Bacterial strains

In this study we used *S. aureus* strain Newman, a human clinical isolate [23] as well as its isogenic knockout mutants SKM12 (Δ(*srtA*)), SKM7 (Δ(*srtB*)), and SKM14, a double (Δ(*srtA*, *srtB*)) mutant [19].

2.3. Murine arthritis and other infectious models

Two independent in vivo animal experiments were performed. Forty mice were subdivided into four groups and inoculated in the tail vein with 6×10^6 *S. aureus* in 200 µl of PBS (phosphate-buffered saline). Weight change, occurrence of arthritis as well as mortality were monitored during the course of experiments, until the mice were sacrificed after 2 weeks. In the second experiment, 13 mice were inoculated with 5×10^6 *S. aureus* wild-type strain Newman, and another 30 mice received an equivalent dose of the isogenic mutant strains, SKM12 Δ(*srtA*), SKM7 Δ(*srtB*), or SKM14 Δ(*srtA*, *srtB*). The mice were monitored for 6 d and then sacrificed. Bacterial load was examined in homogenized kidneys and joints that were excised postmortem from infected animals. Contra-lateral limb joints were used for histopathological examination. Serum samples from each infected mouse were obtained for cytokine analyses. The results of the two experiments were pooled. Permission for animal research was obtained from the Ethics Animal Research Committee of the Göteborg University.

2.4. Bacteriologic examination of infected animals

In the second experiment, mice were inoculated intravenously with 5×10^6 colony-forming units (CFU) of *S. aureus* and sacrificed 6 d after the inoculation. The talocrural and radiocarpal joints of the right pair of extremities were dissected aseptically, and samples were obtained using cotton sticks and were cultured on blood agar plates for 24 h. Bacterial load $\geq 10^4$ in the joints was set to 10^4 CFU. The kidneys were aseptically removed, homogenized and diluted to appropriate concentrations. One hundred microliter of the homogenate was transferred to blood agar plates and incubated for 24 h.

2.5. Histopathological examination

The left pairs of limbs were obtained at the day of sacrifice i.e. 6 d after the inoculation with 5×10^4 *S. aureus* strain Newman or its isogenic mutants. Tissue sections were stained with hematoxylin and eosin. The joints were studied by a blinded examiner (I.-M.J). Synovial hypertrophy was defined as a synovial membrane thickness of >2 cell layers [24]. Histologic scoring was used to quantitate the degree of synovial hypertrophy and degradation of cartilage and bone. Scores were set at 1 point for mild, 2 points for moderate, and 3 points for severe synovitis as well as erosive joint damage [25].

2.6. Analysis of IL-6

Serum samples from mice participating in the second in vivo experiment were used to analyze circulating interleukin-6 (IL-6) levels. 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 [24,26].

2.7. Statistical analyses

Statistical evaluation was accomplished using the Mann–Whitney *U* test or Fischer's exact test. Survival data were analyzed with the Kaplan–Meier log rank test.

All values are reported as median and interquartile range (IQR). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical course of infection

NMRI mice were inoculated with 6×10^6 CFU of staphylococci, either the human clinical isolate strain Newman or its isogenic variants SKM12 (Δ(*srtA*)), SKM7 (Δ(*srtB*)), and SKM14 (Δ(*srtA*, *srtB*)), in 200 µl of PBS into the tail vein. Time to progression of an acute lethal disease was monitored and is reported in Fig. 1. The wild-type Newman strain displayed a significantly higher rate of mortality, with 60% of all animals dead within 11 d. In contrast, neither SKM12 (Δ(*srtA*)) nor SKM14 (Δ(*srtA*, *srtB*)) caused a lethal infection. SKM7 (Δ(*srtB*)) displayed an intermediate phenotype, as 10% of the animals died during the course of this experiment ($P = 0.02$) (Fig. 1). In the second experiment, mice were inoculated with 5×10^6 CFU of staphylococci. Two mice inoculated with the wild-type died before termination of the experiment at day 6, and of the Δ(*srtA*)-, and Δ(*srtB*)-

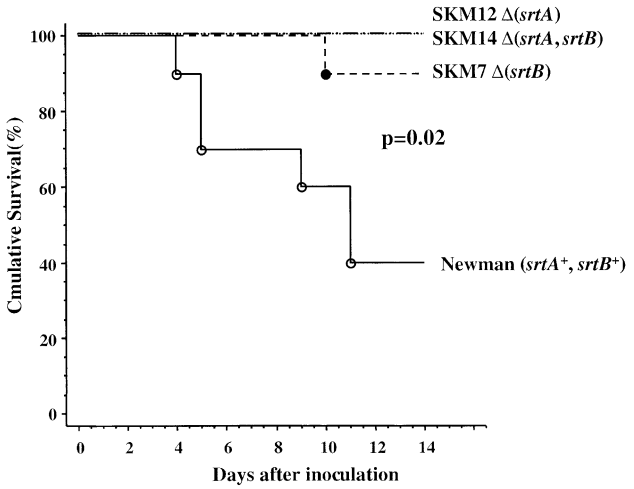

Fig. 1. Cumulative survival of NMRI mice inoculated with 6×10^6 CFU of *S. aureus*. *S. aureus* wild-type strain Newman (*srtA*$^+$, *srtB*$^+$) ($n = 10$) and its isogenic Δ(*srtA*) (SKM12) ($n = 10$), Δ(*srtB*) (SKM7) ($n = 10$) or Δ(*srtA*, *srtB*) (SKM14) ($n = 10$) mutant strains were used for injection. Data shown are the percent of animals injected intravenously and monitored for a period of 14 d.

infected animals, one mouse each died. Again no deaths were registered in the group inoculated with the double mutant Δ(*srtA*, *srtB*) strain.

Mice infected with *S. aureus* wild-type suffered a pronounced weight loss of 18% (Fig. 2). In contrast, mice infected with Δ(*srtA*) and Δ(*srtA*, *srtB*) strains showed no weight loss. The Δ(*srtB*) variant showed an intermediate phenotype (12%, $P < 0.0001$). After 1 week of infection, the decrease in weight of wild-type-inoculated mice was 26%, and for the Δ(*srtB*) mutant-inoculated group, the weight decrease was 19% ($P = 0.007$). Again, the Δ(*srtA*) (4.6%) and Δ(*srtA*, *srtB*) (2%) strains showed very little weight loss (Fig. 2).

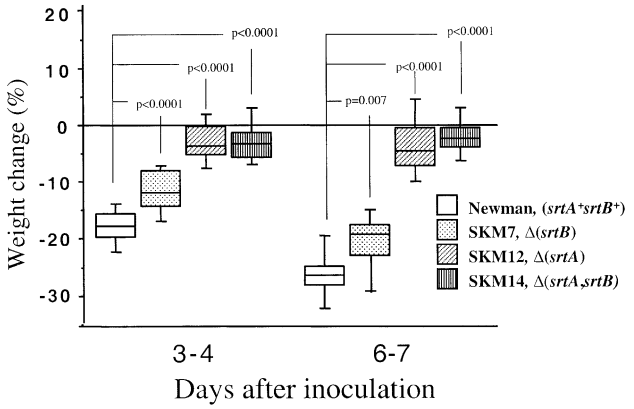

Fig. 2. Changes in body weight of NMRI mice inoculated with $5–6 \times 10^6$ CFU of *S. aureus*. *S. aureus* wild-type (*srtA*$^+$, *srtB*$^+$) ($n = 23$) and its isogenic Δ(*srtA*) (SKM12) ($n = 20$), Δ(*srtB*) (SKM7) ($n = 20$) or Δ(*srtA*, *srtB*) (SKM14) ($n = 20$) mutant strains were used for injection. Data shown are median and interquartile range for each group of mice. Data from the two experiments are pooled. *P* values are those of comparisons between the wild-type strain and any of the mutant strains.

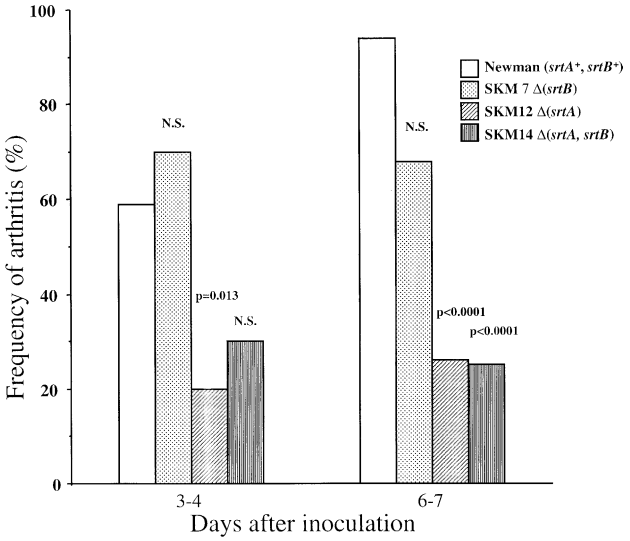

Fig. 3. Frequency of arthritis in NMRI mice inoculated with $5–6 \times 10^6$ CFU of *S. aureus*. *S. aureus* wild-type strain Newman (*srtA*$^+$, *srtB*$^+$) ($n = 23$) and its isogenic Δ(*srtA*) (SKM12) ($n = 20$), Δ(*srtB*) (SKM7) ($n = 20$) or Δ(*srtA*, *srtB*) (SKM14) ($n = 20$) mutant strains were used for injection. Data shown are median and interquartile range for each group of mice. Data from the two experiments are pooled. Comparisons were made using the Mann–Whitney *U* test. *P* values are those of comparisons between the wild-type and any of the sortase mutant strains. N.S., not significant.

3.2. Infectious arthritis

Mice infected with *S. aureus* wild-type strain Newman suffered from infectious arthritis within 1 week (94% of the mice, $n = 18$) (Fig. 3). The Δ(*srtB*) variant gave rise to arthritis in 68% of the mice ($n = 19$; N.S.), whereas only 26% and 25% of mice infected, respectively, with the Δ(*srtA*) ($n = 19$; $P < 0.0001$) or the double mutant Δ(*srtA*, *srtB*) strain ($n = 20$; $P < 0.0001$) showed signs of arthritis (Fig. 3). While early during infection no significant changes in the severity of arthritis could be observed, at the 6- to 7-day time interval, all mice infected with the sortase mutants displayed significantly lower clinical severity of arthritis. The Δ(*srtB*) mutant-inoculated mice had a median arthritis index of 2.0, that of Δ(*srtA*)-inoculated mice was 0, as was that of Δ(*srtA*, *srtB*)-inoculated mice. In contrast, wild-type-inoculated mice displayed a median arthritis index score of 2.0 (Fig. 4).

3.3. Induction of the inflammatory response in infected mice

Serum levels of IL-6 were significantly lower in mice infected with any of the sortase mutant strains than in mice inoculated with wild type. On day 6 after inoculation the median level of IL-6 was 3900 pg/ml (IQR, 1687–4262 pg/ml; $P = 0.03$) for Δ(*srtB*)-inoculated mice, 500 pg/ml (IQR, 400–731; $P = 0.0002$) for Δ(*srtA*)-inoculated mice, and 325 pg/ml (IQR, 100–450 pg/ml; $P = 0.0002$) for the double Δ(*srtA*, *srtB*) mutant-inoculated mice, compared to 16 000 pg/ml (IQR, 4100–16 000 pg/ml) for wild-type strain. These results suggest that the inflammatory response

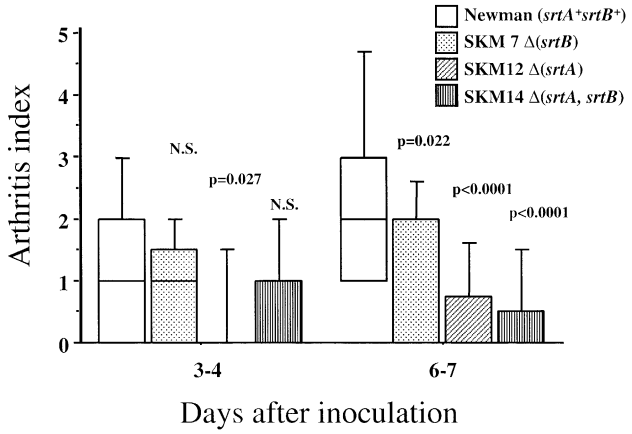

Fig. 4. Severity of arthritis in NMRI mice inoculated with 5–6 × 10^6 CFU of *S. aureus*. *S. aureus* wild-type strain Newman (*srtA*$^+$, *srtB*$^+$) (n = 23) and its isogenic Δ(*srtA*) (SKM12) (n = 20), Δ(*srtB*) (SKM7) (n = 20) or Δ(*srtA*, *srtB*) (SKM14) (n = 20) mutant strains were used for injection. Data shown are median and interquartile range for each group of mice. Data from the two experiments are pooled. Comparisons were made using the Mann–Whitney *U* test. *P* values are those of comparisons between the wild-type and any of the sortase mutant strains. N.S., not significant.

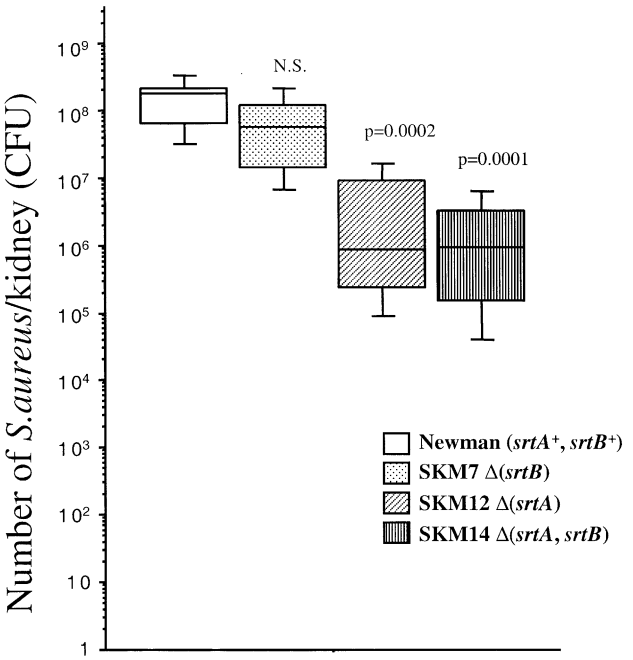

Fig. 5. Establishment of renal abscesses in NMRI mice inoculated with 5 × 10^6 CFU of *S. aureus*. *S. aureus* wild-type strain Newman (*srtA*$^+$, *srtB*$^+$) (n = 11) and its isogenic Δ(*srtA*) (SKM12) (n = 9), Δ(*srtB*) (SKM7) (n = 9) or Δ(*srtA*, *srtB*) (SKM14) (n = 10) mutant strains were used for injection. Animals were sacrificed on day 6, and kidneys were excised and homogenized. The homogenate was plated on blood agar plates and staphylococci counted after incubation and colony formation. Data shown are median and interquartile range for each group of mice. Comparisons were made using the Mann–Whitney *U* test. *P* values are those of comparisons between the wild-type and any of the sortase mutant strains. N.S., not significant.

is diminished during infection with staphylococci that lack either one of the two sortase genes.

3.4. Histopathological analysis of staphylococcal infection

The left pairs of limbs from the second experiment were excised for histopathological examination. Mice inoculated with wild-type strain displayed synovitis with a median score of 2.0 (IQR, 1.25–3.0); also Δ(*srtB*)-inoculated mice displayed a synovitis median score of the same degree 2.0 (IQR, 1.0–3.0); both Δ(*srtA*)-inoculated mice and the double Δ(*srtA*, *srtB*) mutant-inoculated mice had a median synovitis score of 0 (IQR, 0–2.25 and 0–2.0, respectively). The severity of erosivity displayed a median score of 1.0 (IQR, 1.0–2.0) for the wild-type-inoculated mice. The Δ(*srtB*)-inoculated mice's median score was 2.0 (IQR, 0.75–3.0). The Δ(*srtA*) and double mutant both achieved the median severity of erosivity of 0 (IQR, 0–3.0 and 0–1, respectively). The observed differences between wild-type-inoculated and mutant-inoculated groups in this experiment were not statistically significant.

3.5. Staphylococcal persistence in host tissue

Culture samples were obtained from kidneys and from a pair of joints (talocrural and radiocarpal), on day 6 after inoculation with 5 × 10^6 *S. aureus*. Growth of *S. aureus* in joints was found in 10 out of 11 mice (91%) inoculated with wild-type strain, and in 89%, 56%, and 70% of the mice inoculated with Δ(*srtB*), Δ(*srtA*), and double mutant, respectively. Quantification of the bacteria in the joints revealed that mice inoculated with the wild-type strain had a median CFU of 1500 (IQR, 271–10 100; n = 11) in the joints; Δ(*srtB*)-inoculated mice, a median of 750 CFU (IQR, 111–3252; n = 9; N.S.); and Δ(*srtA*)-inoculated mice, a median of 50 CFU (IQR, 11–96; n = 9; P = 0.025) and the double mutant-inoculated mice, 91 (IQR, 22–132; n = 10; P = 0.038).

A similar result was obtained when we analyzed staphylococcal persistence in kidneys (Fig. 5). Mice inoculated with wild-type harbored a median of 17.5 × 10^7 *S. aureus*/kidney (IQR, 6.2 × 10^7–21.6 × 10^7), and for Δ(*srtB)*-inoculated mice, the median CFU was 5.5 × 10^7 (IQR, 1.4 × 10^7–11.7 × 10^7; N.S.). For the Δ(*srtA*) mutant, and double mutant-inoculated mice, the growth of staphylococci in kidneys was even more reduced. The Δ(*srtA*)-inoculated mice had a median CFU of 8.5 × 10^5 (IQR, 2.4 × 10^5–91.3 × 10^5; P = 0.0002) and the double mutant-inoculated mice, 9.3 × 10^5 (IQR, 1.5 × 10^5–33.0 × 10^5; P = 0.0001).

4. Discussion

The purpose of this study was to analyze the contributions of sortase A and sortase B to murine arthritis caused by *S. aureus*. SrtA and SrtB recognize specific surface protein substrates [19]. While sortase A is constitutively expressed, *srtB* and the *isd* locus are only transcribed under low iron conditions [18,19]. The low availability of free iron during animal infection (less than 10^{-18} M^{-1}) strongly [22] suggests

that both *srtA* and *srtB* are expressed during murine infection [18]. Previous work established that *srtA* mutants are defective in the anchoring and surface display of about 20 different polypeptides bearing C-terminal sorting signals with an LPXTG motif [12]. This observation correlates with the strong defect in the pathogenesis of staphylococcal mutants lacking *srtA* during the pathogenesis of murine arthritis [21]. Sortase A and cell wall anchored surface proteins with an LPXTG motif are essential for the establishment of murine arthritis and contribute to the pathogenesis of acute lethal infections as well as the formation of renal abscesses [12,21].

Sortase B recognizes the surface protein substrate IsdC, which carries an NPQTN motif sorting signal [19]. To the best of our knowledge, this is the only surface protein substrate for SrtB. The precise role of IsdC in the physiology of staphylococcal infections is not yet known; however, preliminary data suggest that *isdC* and the *isd* locus may be involved in the transport of heme-iron across the bacterial envelope [18]. What then, if any, is the role of *srtB*, in establishing murine arthritis? Compared with mice that were infected with wild-type strain Newman, Δ(*srtB*), Δ(*srtA*), and double Δ(*srtA*, *srtB*) mutant strains showed better survival rates during our experiment. Staphylococci lacking *srtB* displayed a small defect in the establishment of murine arthritis, as evidenced by smaller weight losses. When considering the histopathological analysis of mouse joints, which did not show significant differences with respect to the erosivity of the infection, we conclude that *srtB* does not play a prominent role during the pathogenesis of destructive murine arthritis. One week following inoculation with staphylococci, there was a significant difference ($P = 0.02$) in clinical severity of arthritis between Δ(*srtB*) mutant and wild-type-inoculated mice. This finding correlates with the significantly lower increases in levels of circulating IL-6, a reliable measure of systemic inflammatory drive, in Δ(*srtB*) mutant-inoculated mice compared with animals that had been infected with wild-type. Finally, clinical and histopathological examination of mice infected with SKM17 revealed that the combination of two sortase deletions, Δ(*srtA*, *srtB*), did not lead to a further aggravation of virulence defects than with the Δ(*srtA*) mutant strain.

In conclusion, both sortase A and sortase B are virulence factors participating in the establishment and persistence of *S. aureus* infections. While the critical impact of sortase A for the surface display of adhesins and immune modulatory molecules is well established, the role of sortase B requires further study.

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