

# Activation of Plasminogen by Staphylokinase Reduces the Severity of *Staphylococcus aureus* Systemic Infection

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**Background.** Staphylokinase (SAK) is produced by the majority of *Staphylococcus aureus* strains. It is an extracellular protein that activates the conversion of human plasminogen (plg) to plasmin. The role played by SAK in staphylococcal infection is unclear.

**Methods.** Wild-type *S. aureus* strain LS-1, which lacks the ability to produce SAK, was modified by an insertion of the *sak* gene into its chromosome. The *sak* gene was integrated in 2 forms—(1) linked to its own promoter and (2) fused to the promoter of the protein A gene—which resulted in the overexpression of SAK. SAK is highly specific for human plg and exhibits almost no activity toward murine plg. To investigate the role played by SAK in a murine infection model, human plg transgenic mice and their wild-type counterparts were inoculated intravenously with congenic *S. aureus* strains differing in SAK production.

**Results.** Human plg transgenic mice inoculated with SAK-expressing strains displayed significantly reduced mortality, less weight loss, and lower bacterial loads in kidneys than did the wild-type mice. No difference in the severity of sepsis was observed between transgenic and wild-type mice infected with a SAK-deficient strain.

**Conclusions.** The results suggest that expression of SAK followed by activation of plg alleviates the course of *S. aureus* sepsis.

Nowadays, *Staphylococcus aureus* is one of the major pathogens of the world, causing a wide range of infectious diseases. Despite proper treatment, mortality for some diseases might even exceed 50% [1]. Therefore, the need for a better understanding of staphylococcal virulence and infection mechanisms remains an urgent issue.

Staphylokinase (SAK) is an extracellular protein ex-

pressed by most strains of *S. aureus*. It forms a complex with human plasminogen (plg), promoting its activation to plasmin [2, 3]. It has been shown that SAK is a highly specific activator of human plg and has only limited activity toward murine plg [4]. The genes for SAK from different strains display minimal differences that do not affect the capacity of SAK to activate plg [5–7]. Expression of SAK is positively regulated by the accessory gene regulator Agr [8] and is negatively regulated by the staphylococcal accessory regulator SarA [9].

The role played by SAK expression in staphylococcal infection remains unresolved. Patients infected with SAK-deficient isolates were 3–4 times more likely to have lethal bacteremia than patients whose infecting isolates produced high levels of SAK [10]. However, it is known that SAK-producing staphylococci acquire a cell-associated plasminlike activity, potentially supporting bacterial invasion into host tissues via plasmin-induced proteolysis [11]. *S. aureus* is able to immobilize plg on the cell surface [12–14]. Consequently, plg bound to bacteria can be activated by host plg activators

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**Table 1. Bacterial Strains, Plasmids, and Primers**

Name	Genotype	Phenotype	Relevant properties or sequence	Source or reference
<i>Staphylococcus aureus</i> strains				
NCTC8325		SAK <sup>+</sup>	<i>sak</i> and <i>spa</i> genes amplified by PCR	Laboratory stock
CYL316		SAK <sup>-</sup>	RN4220 (pYL112D19); host for integrating pCL84 in <i>geh</i> gene	[19]
CYL316::pCL84	<i>geh</i> ::pCL84	SAK <sup>-</sup>	pCL84 integrated in CYL316	This study
CYL316::pJT2	<i>geh</i> ::pJT2	SAK <sup>+</sup>	pJT2 (pCL84 <i>sak</i> ) integrated in CYL316	This study
CYL316::pJT4	<i>geh</i> ::pJT4	SAK <sup>+</sup>	pJT4 (pCL84 <i>spa-sak</i> ) integrated in CYL316	This study
LS-1		SAK <sup>-</sup>	Wild type	[20]
LS-1EP	<i>geh</i> ::pCL84	SAK <sup>-</sup>	pCL84 integrated into LS-1	This study
LS-1 <i>sak</i>	<i>geh</i> ::pJT2	SAK <sup>+</sup>	pJT2 (pCL84 <i>sak</i> ) integrated into LS-1	This study
LS-1 <i>spa-sak</i>	<i>geh</i> ::pJT4	SAK <sup>++</sup>	pJT4 (pCL84 <i>spa-sak</i> ) integrated into LS-1	This study
<i>Escherichia coli</i> strains				
XL-1 blue	F <sup>+</sup> Tn 10proA <sup>+</sup> B <sup>-</sup> lacZ(lacZ)M15(recA)gyrA96(Nal <sup>r</sup> ) <i>thihsdR17(rk<sup>-</sup>mk<sup>+</sup>)supE44relA/lac</i>		Host for recombinant plasmids	New England Biolabs
Plasmids				
pCU1		Ap <sup>r</sup> , Cm <sup>r</sup>	<i>S. aureus/E. coli</i> shuttle plasmid	[21]
pJT1	<i>sak</i>	Ap <sup>r</sup> , Cm <sup>r</sup> ; SAK <sup>+</sup>	pCU1 containing the <i>sak</i> gene and flanking sequence	This study
pJT3	<i>spa-sak</i>	Ap <sup>r</sup> , Cm <sup>r</sup> ; SAK <sup>+</sup>	pCU1 containing a 415-bp <i>spa</i> promoter fragment fused to a promoter-less <i>sak</i> gene	This study
pCL84		Sp <sup>8</sup> , Tc <sup>6</sup> ; <i>attP</i> <sub>CL84</sub>	<i>S. aureus</i> single-copy integration plasmid; integrates at $\Phi$ L54 <i>attB</i> in <i>geh</i>	[19]
pJT2	<i>sak</i>	Sp <sup>8</sup> , Tc <sup>6</sup> ; <i>attP</i> <sub>CL84</sub> ; SAK <sup>+</sup>	<i>sak</i> gene and flanking sequence from pJT1 cloned into pCL84	This study
pJT4	<i>spa-sak</i>	Sp <sup>8</sup> , Tc <sup>6</sup> ; <i>attP</i> <sub>CL84</sub> ; SAK <sup>+</sup>	<i>spa-sak</i> from pJT3 cloned into pCL84	This study
Primers				
SAKFlank F	...	...	CGCGGATCCGGTCAAAAAGGTGCTTATTTAATG	This study
SAKFlank R	...	...	CCGGAAATTCATATACTCTCATCAAGAAAAAAC	This study
SpaHind F	...	...	CCCAAGCTTCTATTACGCAAGTGTGCTGT	This study
SpaNco1 R	...	...	CGCGGATCCCACATGGATTAATACCCCTGT	This study
SAKNco1 F	...	...	CATGCCAATGGCACTCAAAAAGAGTTTATTTTAACT	This study
SAKEcoR R	...	...	CCGGAAATTCATATACTCTCATCAAGAAAAAAC	This study
SpaBamH F	...	...	CGCGGATCCCCTATTACGCAAGTGTGCTGATTT	This study
SAKEcoR R	...	...	CCGGAAATTCATATACTCTCATCAAG	This study
SAKstop R	...	...	CCGGAAATTCATTTCTTTCTATAATAACCTTTGT	This study

**NOTE.** Ap<sup>r</sup>, resistant to ampicillin; bp, base pair; Cm<sup>r</sup>, resistant to chloramphenicol; PCR, polymerase chain reaction; SAK, staphylokinase; Sp<sup>8</sup>, resistant to spectinomycin; Tc<sup>6</sup>, resistant to tetracycline. Underscoring in primer sequences indicates recognition sites of restriction enzymes.

**Table 2. Experimental Protocols**

Bacterial load per mouse	Experiment duration, days	Human plasminogen transgenic mice			Wild-type C57BL6/J mice		
		LS-1 <i>EP</i>	LS-1 <i>sak</i>	LS-1 <i>spa-sak</i>	LS-1 <i>EP</i>	LS-1 <i>sak</i>	LS-1 <i>spa-sak</i>
$2 \times 10^7$ – $2.5 \times 10^7$ CFUs	21	33	29	33	18	12	17
$6 \times 10^7$ CFUs	14	10	11	12	9	8	10
$1 \times 10^7$ CFUs	7	...	...	10	...	...	6

**NOTE.** The no. of mice used for experiments with different bacterial loads and *Staphylococcus aureus* strains are shown. CFUs, colony-forming units.

[15] and by the endogenous activator SAK. Apart from its ability to activate plg, SAK inhibits the bactericidal activity of human  $\alpha$ -defensins [16] and potentially protects bacteria from phagocytosis by cleaving host opsonins [17].

In the present study, we investigated the role played by SAK in staphylococcal infection. We developed a panel of congenic *S. aureus* strains differing in SAK production. Transgenic mice expressing human plg were used for experimental infections [18]. Production of SAK decreased the severity of systemic staphylococcal infection—an effect that was clearly related to the SAK-mediated activation of human plg in the transgenic mice.

## METHODS

**Bacterial strains and growth media.** Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* was grown in Luria broth or on Luria agar supplemented with ampicillin (100  $\mu$ g/mL) or spectinomycin (25  $\mu$ g/mL). *S. aureus* strains were grown in trypticase soy broth (TSB) or on trypticase soy agar (TSA) containing chloramphenicol (10  $\mu$ g/mL) or tetracycline (2  $\mu$ g/mL). For the lipase assay, TSA containing 1% Tween 20 and 0.1%  $\text{CaCl}_2$  was used.

**Cloning and expression of the sak gene in *S. aureus* LS-1.** Standard procedures were used for genetic manipulation [22, 23]. The oligonucleotides are listed in Table 1. The *sak* gene, including the promoter region, was amplified from *S. aureus* 8325 genomic DNA by means of primers SAKflankF and SAKflankR, incorporating a *Bam*HI site and an *Eco*R1 site, respectively. Amplimers were cut with the appropriate enzymes, ligated with pCL84 DNA cleaved with the same enzymes, and transformed into *E. coli* XL-1Blue selecting for spectinomycin resistance, forming pJT2.

Oligonucleotides SpaHindF and SpaNco1R were designed to amplify the protein A gene *spa* promoter and ribosome binding site [24] and to modify the *spa* ATG codon so that it would be incorporated into an *Nco*I site. Oligonucleotide SAKNco1F was designed to modify the ATG codon of the *sak* gene to include an *Nco*I site and was combined with primer SAKecoR. The *spa* promoter and *sak* gene fragments were amplified with the respective primer pairs; cleaved with *Hind*III, *Nco*I, and

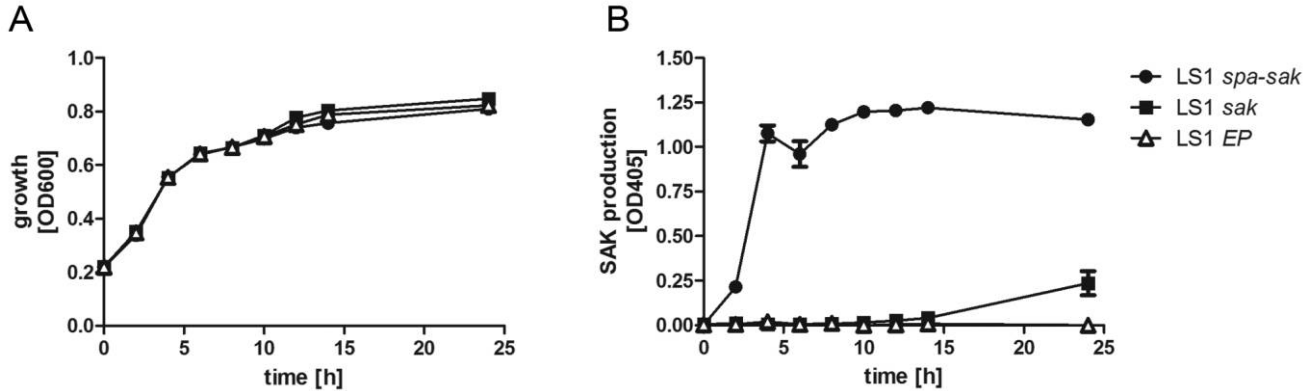
*Eco*R1, as appropriate; and ligated with *Hind*III- and *Eco*R1-cleaved pCU1, forming plasmid pJT3. This was used as a template for polymerase chain reaction (PCR) with primers SpaBamHF and SAKecoRR. The amplimer was cut with *Bam*HI and *Eco*R1 and was ligated with *Bam*HI- and *Eco*R1-cut pCL84, forming pJT4.

Plasmids pJT2 and pJT4 were integrated into the lipase gene by means of the phage L54a attachment site carried by pCL84 and expression of phage integrase by recipient *S. aureus* strain CYL316. Insertion of the vector inactivated lipase production, which was confirmed by a lipase plate assay. The integrated plasmids were transduced into strain LS-1 by means of phage 85, which selected for tetracycline resistance.

The integrity of the *sak* gene and the *spa-sak* fusion in strains LS-1*sak* and LS-1*spa-sak* was assessed by PCR (using primers previously utilized for cloning) and Southern hybridization [23, 25].

**Detection of SAK production.** Overnight cultures of *S. aureus* strains (1 mL of TSB at 37°C) were washed with phosphate-buffered saline (PBS), resuspended in 10 mL of fresh TSB, and incubated at 37°C. At different time points, growth of bacteria was measured on the basis of the optical density read at 600 nm ( $\text{OD}_{600}$ ), and samples of culture were taken. Samples were centrifuged (4000g for 10 min at 4°C), and the supernatants were collected for analysis of SAK activity. Human Glu-plg ( $2 \times 10^{-7}$  mol/L in 50 mmol/L Tris-HCl [pH 7.4]) was mixed with supernatants and incubated at 37°C for 10 min, to allow plasmin formation. The plasmin-specific chromogenic substrate H-D-Val-Leu-Lys-Paranitroanilide (S-2251; Chromogenix;  $4 \times 10^{-4}$  mol/L) was then added. After 60 min, plasmin-dependent hydrolysis of the S-2251 was measured at  $\text{OD}_{450}$ . The concentration of SAK in the supernatants was represented by the absorbance value.

**Mice.** Human plg transgenic mice (hereafter, h-plg mice) were used [18]; these mice express human plg ( $Tg^{+/-}$ ) under the control of albumin gene regulatory sequences. They were backcrossed with C57BL6/J mice (Charles River). For genotyping of offspring, genomic DNA was prepared from tail biopsy samples, and h-plg mice and wild-type mice were identified by PCR amplification of the plg gene [18]. Mice were



**Figure 1.** In vitro growth (A) and staphylokinase (SAK) production (B) in cultures of congenic *Staphylococcus aureus* strains (LS-1EP, LS-1sak, and LS-1spa-sak). For SAK activity, data are shown as means  $\pm$  standard errors for a duplicate assay. OD, optical density.

housed in the animal facility of the Department of Rheumatology and Inflammation Research, Sahlgrenska Academy, Gothenburg University, under standard conditions of temperature and light (10 mice in a cage) and were fed standard laboratory chow and water ad libitum. Mice were used for experimental infection at the age of 6–8 weeks. The study was approved by the Ethic Committee of Sahlgrenska Hospital, and animal experimentation guidelines were followed.

**Preparation of inocula.** Bacterial batches used for infection were prepared from cells grown for 24 h on horse blood agar plates. Cells were harvested, resuspended in PBS supplemented with 5% bovine serum albumin and 10% dimethyl sulfoxide, and stored frozen in 1-mL portions at  $-20^{\circ}\text{C}$ . Bacterial counts of frozen bacterial batches were checked before storage to facilitate preparation of bacterial inocula with the required bacterial load. Before inoculation of mice, suspensions were thawed, washed in PBS, and diluted in PBS to the appropriate concentration. Viable counts of the leftover inocula were performed.

**Activation of fibrinolysis in mouse plasma by tissue plg activator and SAK.** Spontaneous plasmin activity and tissue plg activator (tPA)-induced plasmin activity were determined by hydrolysis of a plasmin-specific chromogenic substrate (S-2251) in plasma samples from h-plg mice ( $n = 3$ ) and wild-type mice ( $n = 3$ ). Pooled plasma samples were diluted 2-fold in 50 mmol/L Tris-HCl (pH 7.4) and mixed with recombinant tPA (Alteplase; Boehringer Ingelheim; final concentration, 900 U/mL) or buffer alone and incubated for 10 min at  $37^{\circ}\text{C}$ . S-2251 ( $4 \times 10^{-4}$  mol/L) was added and incubated for 30 min, and the OD<sub>405</sub> was measured.

SAK-induced plasmin activity was determined by S-2251 hydrolysis in plasma from h-plg mice ( $n = 2$ ) and wild-type mice ( $n = 2$ ). Pooled plasma samples were diluted 10-fold in 50 mmol/L Tris-HCl (pH 7.4) and mixed with recombinant SAK (sakSTAR; provided by Prof D. Collen, Center for Transgene

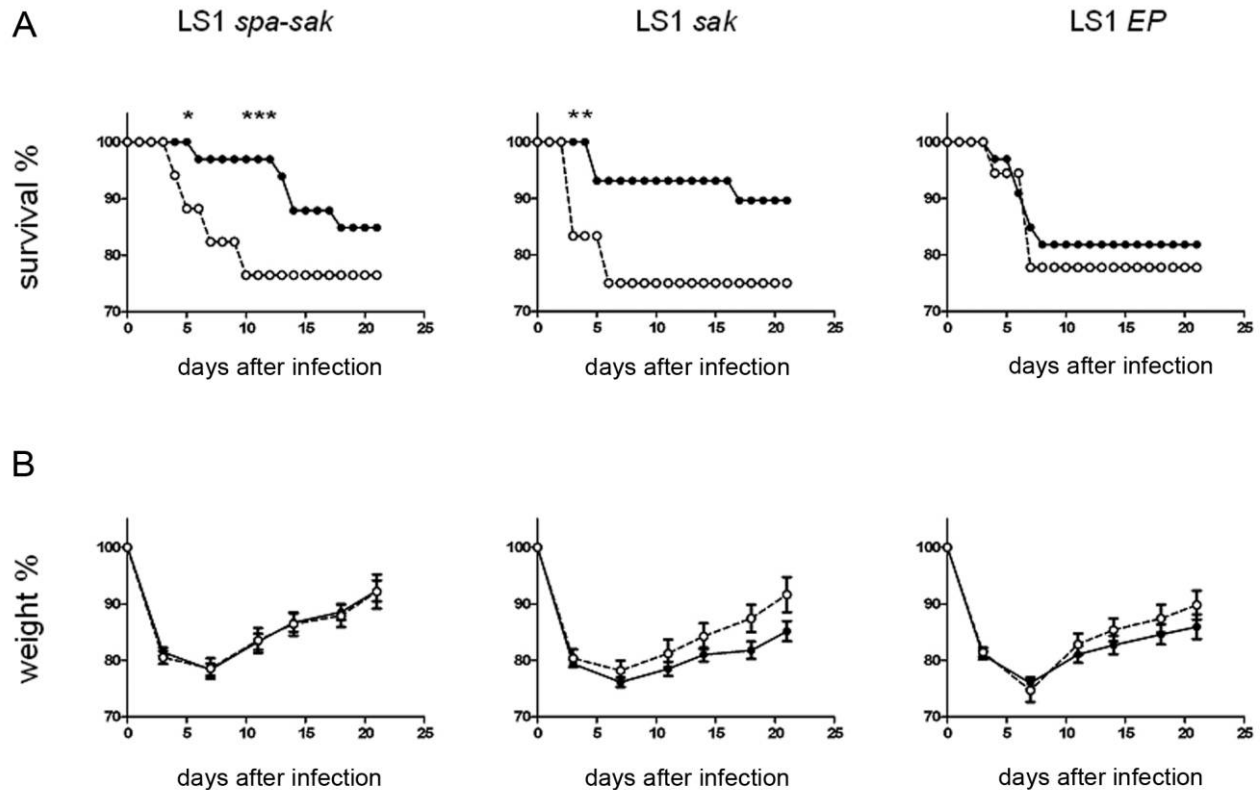
Technology and Gene Therapy, Flanders Interuniversity Institute of Biotechnology, Leuven, Belgium; final concentration, 50  $\mu\text{g}/\text{mL}$ ) or buffer alone and incubated for 10 min at  $37^{\circ}\text{C}$ . S-2251 ( $4 \times 10^{-4}$  mol/L) was added and incubated for 30 min, and the OD<sub>405</sub> was measured.

To confirm the ability of SAK to activate fibrinolysis in h-plg mice, an in vitro clot lysis assay was performed. Plasma collected from h-plg mice ( $n = 3$ ) and wild-type mice ( $n = 3$ ) (50  $\mu\text{L}$ ) was mixed with 10  $\mu\text{L}$  of 0.1 mol/L CaCl<sub>2</sub>, 0.2 National Institutes of Health units of thrombin, and 40  $\mu\text{L}$  of supernatant from overnight culture of *S. aureus* LS-1spa-sak or LS-1EP and incubated at  $37^{\circ}\text{C}$  for 90 min, and clot formation and lysis was measured at OD<sub>340</sub>. The percentage of the clot that remained after 2 h was recorded.

**Experimental infection.** Congenic bacterial strains LS-1EP, LS-1sak, and LS-1spa-sak were used for in vivo experiments. Each set of experiments contained both h-plg mice and their wild-type littermates (Table 2).

In experiments 1–3, mice were injected intravenously with  $2 \times 10^7$ – $2.5 \times 10^7$  colony-forming units (CFUs) of *S. aureus* LS-1EP, LS-1sak, or LS-1spa-sak in 200  $\mu\text{L}$  of PBS. Clinical signs of infection were monitored until day 21. In experiment 4, mice were injected intravenously with  $6 \times 10^7$  CFUs of each strain, and clinical signs of infection were monitored until day 14. In experiment 5, mice were injected intravenously with  $1 \times 10^7$  CFUs of *S. aureus* LS-1spa-sak, and clinical signs of infection were monitored until day 7. Clinical assessment included evaluation of general appearance, alertness, skin abnormalities, changes in mouse body weight, and signs of arthritis [26]. At the termination of experiments, blood was collected for tPA and plg activator inhibitor 1 (PAI-1) analysis, and kidneys were extracted for assessment of bacterial load.

**Bacteriological examination of infected animals.** Both kidneys from each mouse were aseptically removed and mechanically homogenized, diluted in PBS, spread on horse blood



**Figure 2.** Survival (A) and weight loss (B) among human plasminogen transgenic mice (h-plg mice) and wild-type mice after intravenous inoculation with  $2 \times 10^7$ – $2.5 \times 10^7$  colony-forming units of congenic *Staphylococcus aureus* strains differing in staphylokinase production (LS-1*EP*, LS-1*sak*, and LS-1*spa-sak*). White circles indicate wild-type mice, and black circles indicate h-plg mice. Data are shown as means  $\pm$  standard errors. Survival was compared using the Fisher exact test if the experimental groups comprised <10 animals and using the  $\chi^2$  test for larger groups; weight loss was compared using the Mann-Whitney *U* test. \**P* < .05.

agar plates, and incubated for 24 h at 37°C. CFUs were then counted.

**Determination of serum tPA and PAI-1 levels.** Levels of tPA and PAI-1 in serum were measured using mouse tPA total antigen enzyme-linked immunosorbent assay (Innovative Research) and mouse PAI-1 total antigen assay (Innovative Research), respectively.

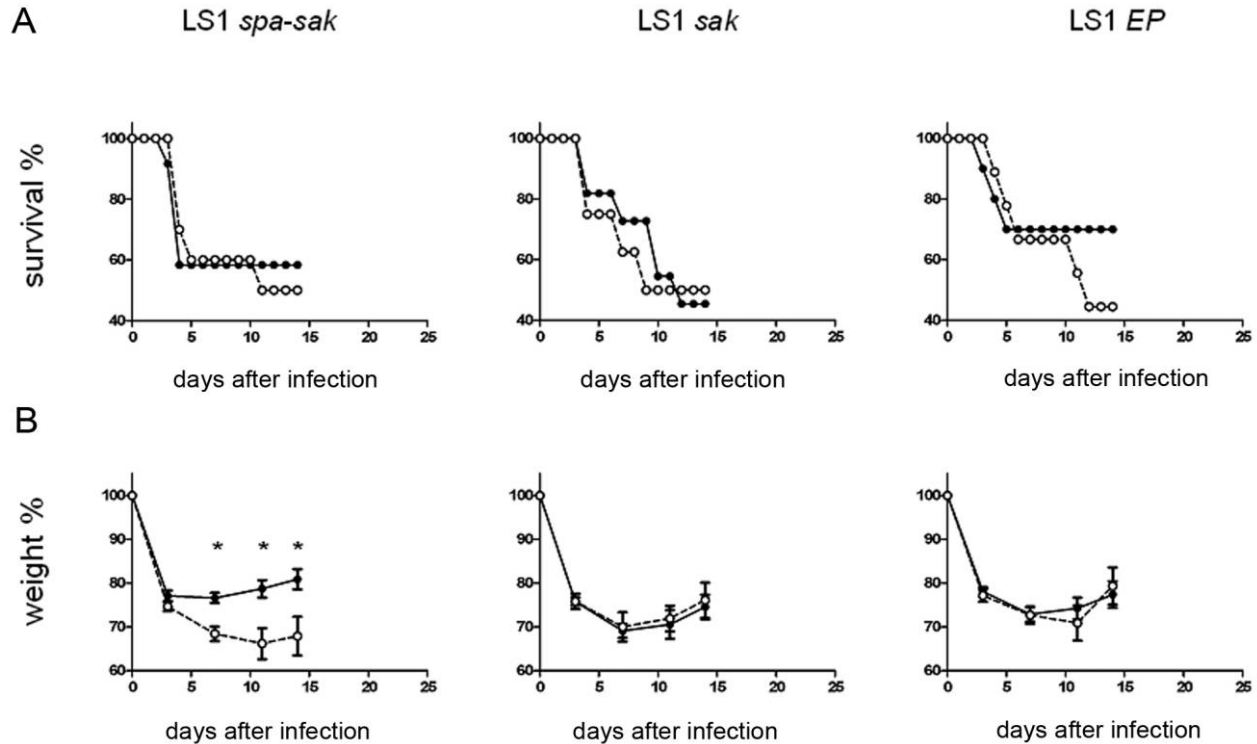
**Statistical analysis.** Comparison of continuous parameters (weight loss and clinical arthritis index) between the groups was performed using the Mann-Whitney *U* test. Survival and frequency of arthritis were compared using the Fisher exact test if the experimental groups comprised <10 animals and using the  $\chi^2$  test for larger groups. For all comparisons, differences were considered significant at *P* < .05. Analyses were performed using Prism software (version 5.01; GraphPad).

## RESULTS

**Construction of *S. aureus* LS-1 expressing SAK.** Strain LS-1 does not express SAK. To generate congenic variants that express SAK, the *sak* gene was cloned into the integrating vector pCL84. In one construct (LS-1*sak*), the *sak* gene was expressed

from its own promoter. In the other, *sak* was fused to the *spa* promoter (LS-1*spa-sak*). The empty vector was used as a control (LS-1*EP*). The pCL84 plasmids were transformed into CYL316, where they integrated into the *geh* gene mediated by phage L54a integrase. The integrants were then transduced into LS-1 by means of phage 85. The integrity of the *sak* gene and the *spa-sak* fusion in strains LS-1*sak* and LS-1*spa-sak* was confirmed by PCR and Southern hybridization.

**SAK production by *S. aureus* LS-1.** The growth of the *S. aureus* LS-1 transductants and their expression of SAK were measured. No difference in rates of growth was observed when the strains were grown in vitro (Figure 1A). Measurement of SAK production revealed that LS-1*spa-sak* started secreting SAK during the early exponential growth phase, whereas the LS-1*sak* strain secreted small, barely detectable amounts of SAK during the exponential growth phase and only started secreting larger amounts of SAK after entering the stationary phase. No secretion of SAK by LS-1*EP* was detected at any phase of growth. LS-1*spa-sak* produced almost 5-fold more SAK than did LS-1*sak* (Figure 1B). The kinetics of SAK expression by LS-1*sak* mimicked the SAK kinetics predicted for clinical strains.



**Figure 3.** Survival (A) and weight loss (B) among human plasminogen transgenic mice (h-plg mice) and wild-type mice after intravenous inoculation with  $6 \times 10^7$  colony-forming units of congenic *Staphylococcus aureus* strains differing in staphylokinase production (LS-1EP, LS-1sak, and LS-1spa-sak). White circles indicate wild-type mice, and black circles indicate h-plg mice. Data are shown as means  $\pm$  standard errors. Survival was compared using the Fisher exact test if the experimental groups comprised  $<10$  animals and using the  $\chi^2$  test for larger groups; weight loss was compared using the Mann-Whitney *U* test. \**P* < .05.

**Staphylococcal infection.** *S. aureus* LS-1sak, LS-1spa-sak, and LS-1EP were injected intravenously in h-plg and wild-type mice at a mild bacteremia dose ( $2 \times 10^7$ – $2.5 \times 10^7$  CFUs per mouse per strain) (Table 2). Data were pooled from 3 independent experiments (Figure 2). At this inoculum, survival of mice was between 75% and 90%. Improved survival was observed in h-plg mice infected with either of the SAK-expressing strains (LS-1sak or LS-1spa-sak), compared with wild-type mice. The difference in survival was most pronounced on days 4–5 for LS-1sak and up to day 12 for LS-1spa-sak. There was no difference in survival of wild-type and h-plg mice infected with LS-1EP (Figure 2A). In addition, analysis with respect to the infecting strain showed that h-plg mice infected with SAK-producing strains had improved survival compared with the h-plg mice infected with the SAK-deficient strain LS-1EP. In contrast, SAK production had no effect on the survival of wild-type mice.

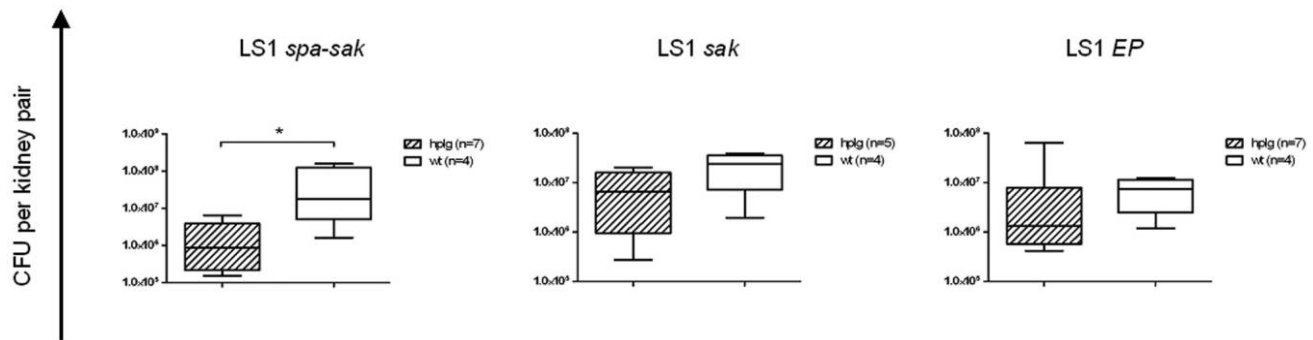
During the first 7 days of infection, mice lost up to 25% of body weight. Thereafter, body weight increased. No difference in weight loss was observed between mice infected with SAK-producing and SAK-deficient LS-1 strains (Figure 2B). Weight loss was similar in h-plg and wild-type mice. The frequency

and severity of arthritis was similar in mice infected with SAK-producing and SAK-deficient strains, as well as in h-plg and wild-type mice (data not shown). At termination of the experiments, no difference was observed in bacterial load between mice infected with SAK-producing and SAK-deficient strains or between h-plg and wild-type mice (data not shown).

In an experiment in which severe bacteremia was studied, a higher inoculum of  $6 \times 10^7$  CFUs/mouse was injected in h-plg and wild-type mice (Table 2). The experiment was discontinued on day 14 because of a low survival rate. The level of survival from severe staphylococcal infection was similar in h-plg and wild-type mice. In addition, no difference in survival rate was found between the mice infected with SAK-producing and SAK-deficient strains (Figure 3A).

Mice infected with the high dose of LS-1 lost as much as 35% of their body weight. H-plg mice infected with LS-1spa-sak lost significantly less weight on each day of infection after the third day, compared with wild-type mice infected with the same strain (Figure 3B). No difference in weight loss was observed between h-plg and wild-type mice infected with LS-1sak and LS-1EP.

The staphylococcal load in the kidneys of h-plg and wild-



**Figure 4.** Bacterial loads in the kidneys of human plasminogen transgenic mice (h-plg mice) and wild-type mice 14 days after intravenous inoculation with  $6 \times 10^7$  colony-forming units (CFUs) of congenic *Staphylococcus aureus* strains differing in staphylokinase production (LS-1EP, LS-1sak, and LS-1spa-sak). Data are shown as medians (central lines), interquartile ranges (boxes), and 80% central ranges (whiskers). The Mann-Whitney *U* test was used for statistical analysis. \* $P < .05$ .

type mice was similar after infection with SAK-deficient strain LS-1EP (Figure 4). A lower level of SAK-producing bacteria was observed in h-plg mice than in wild-type mice. This difference was most pronounced in mice infected with the LS-1spa-sak strain.

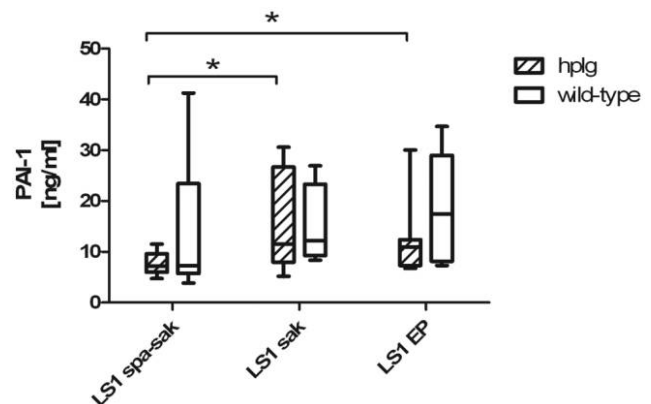
To explore the early events of infection,  $1 \times 10^7$  CFUs of *S. aureus* LS-1spa-sak was injected into h-plg and wild-type mice (Table 2). During the first 7 days of infection, mortality was low (0% in h-plg mice and 17% in wild-type mice). Mice lost ~20% of weight, with no difference between the mouse strains. Both mouse strains developed arthritis of similar frequency and severity. Staphylococcal load in kidneys on day 7 did not differ between h-plg mice and wild-type mice.

**Fibrinolysis in plasma from h-plg mice.** There was no difference in the levels of PAI-1 or tPA between h-plg mice and wild-type mice after staphylococcal infection. However, PAI-1 levels in h-plg mice were inversely related to the amount of SAK production by the infecting strain. Thus, levels of PAI-1 in h-plg mice infected with LS-1spa-sak were significantly lower than those in h-plg mice infected with LS-1sak and in h-plg mice infected LS-1EP (Figure 5).

The amount of active plasmin in plasma from healthy h-plg mice and wild-type mice was compared using an in vitro assay. In the absence of tPA, the amount of spontaneously converted plasmin was similar in all mice. After the addition of human recombinant tPA, the generation of plasmin was enhanced by 21% in the plasma of h-plg mice, compared with that in wild-type mice. Formation of plasmin in plasma from h-plg mice was dramatically enhanced after the addition of recombinant SAK (195% of spontaneous plasmin activity). Plasmin activity in plasma from wild-type mice was barely affected by the addition of SAK (7% increase).

On the basis of the results described above and on the low PAI-1 levels during infection with SAK-producing strains, we

compared the fibrinolytic activity in plasma from h-plg mice with that from wild-type mice in functional assay. Lysis of thrombin-induced plasma clots was performed with culture supernatants of LS-1spa-sak and LS-1EP. Addition of LS-1spa-sak supernatant to the clot formed with h-plg plasma caused efficient lysis (75% lysis after 90 min). In contrast, only 20% of clot was lysed following the addition of the SAK-free LS-1EP supernatant. This difference in clot lysis was not observed with plasma from wild-type mice. These findings demonstrate



**Figure 5.** Serum levels of plasminogen (plg) activator inhibitor 1 (PAI-1) in human plg transgenic mice (h-plg mice) and wild-type mice inoculated with a low dose ( $2 \times 10^7$ – $2.5 \times 10^7$  colony-forming units) of congenic *Staphylococcus aureus* strains differing in staphylokinase production (LS-1EP, LS-1sak, and LS-1spa-sak) on day 21 after infection. The number of h-plg mice analyzed was as follows:  $n = 14$  for LS-1spa-sak,  $n = 11$  for LS-1sak, and  $n = 13$  for LS-1EP. The number of wild-type mice analyzed was as follows:  $n = 9$  for LS-1spa-sak,  $n = 4$  for LS-1sak, and  $n = 6$  for LS-1EP. Data are shown as medians (central lines), interquartile ranges (boxes), and 80% central ranges (whiskers). The Mann-Whitney *U* test was used for statistical analysis. \* $P < .05$ .

that SAK is capable of interacting with human plg of transgenic mice and subsequently activate fibrinolysis.

## DISCUSSION

Despite the availability of modern treatments, infections with *S. aureus* remain a challenge to clinicians [1]. To provide better therapies, a deeper understanding of staphylococcal virulence is needed. To assess the possible role played by SAK in the development of staphylococcal infections, we created *S. aureus* LS-1 congenic strains varying in their level of SAK production. The *sak* gene expressed from its own promoter and the *sak* gene fused to the protein A gene promoter were introduced into the chromosome of the *sak*-negative *S. aureus* strain LS-1. In vitro, the *spa-sak* strain produced ~5-fold more SAK than did the wild-type strain, indicating that the *spa* promoter expression system is of use for expressing *S. aureus* proteins in vitro and in vivo. We have shown that SAK has an ability to activate fibrinolysis in the plasma of h-plg mice as it does in human plasma, indicating that these animals can be used in models for studying the effects of SAK on staphylococcal infections.

Infection with *S. aureus* LS-1EP, which lacks SAK production, resulted in disease of similar severity in h-plg and wild-type mice. This clearly shows that the presence of human plg in h-plg mice does not itself affect the course of infection in the absence of SAK. However, differences in disease severity between h-plg and wild-type mice were apparent when the animals were infected with the SAK-producing strain LS-1*sak* or LS-1*spa-sak*. Given that the bacterial inocula were prepared from the batches with known bacterial counts and the viable counts were corroborated for inocula of each bacterial strain, differences in disease severity were caused by expression of SAK rather than by differences in bacterial load.

We have shown that h-plg mice with mild bacteremia infected with both SAK-producing strains had a higher survival rate than did wild-type mice infected with same strains. In addition, in the severe bacteremia experiment, h-plg mice infected with the LS-1*spa-sak* strain lost less weight and had improved bacterial clearance from the kidneys, compared with infected wild-type mice. Reduced bacteria count in kidneys may reflect the difference in bacterial clearance between the mouse strains. The h-plg mice infected with LS-1*spa-sak* had efficient bacterial elimination and recovered quicker than did the wild-type mice, which exhibited prolonged weight loss and higher bacterial load. Bacterial clearance and weight loss on day 21 was similar during mild bacteremia, despite a marked difference in mortality. One explanation of this fact may be that the sickest animals (with the highest bacterial load and weight loss) were automatically eliminated from both groups by their death. Interestingly, SAK production was of highest importance in the

course of infection induced by a high dose of bacteria, which may be related to the amount of SAK produced in circulation.

PAI-1 acts as an acute-phase protein during systemic staphylococcal infections [27], counteracting the fibrinolytic activity of plasmin [28]. It has been shown that high levels of PAI-1 correlate with a fatal outcome of some infectious diseases [29]. Therefore, a lowered level of PAI-1 in h-plg mice infected with LS-1*spa-sak* further underlines the reduced severity of infection in this group.

Our data indicate that activation of host plg by SAK reduces the virulence of *S. aureus* in the bacteremia model. This is in agreement with our earlier observations in infected humans [10]. Among patients with *S. aureus* bacteremia, there was an inverse correlation between SAK production and clinical outcome. Patients infected with SAK-deficient strains were >4 times more likely to develop lethal bacteremia than those infected with SAK-producing strains [10]. These data suggest that instead of being a virulence factor—which was suggested after purely in vitro experiments—SAK in vivo might be involved in maintaining a symbiotic relationship with the host and preventing systemic infection. One might speculate that activation of host plg would lead to cleavage of the fibrinogen on the *S. aureus* surface. It has been suggested that this surface-bound fibrinogen protects bacteria from phagocytosis [26, 30, 31]. Activation of host plg would also cause degradation of the extracellular matrix used for bacterial adherence [32, 33], resulting in diminished virulence.

In the absence of h-plg, the severity of staphylococcal infection was similar, indicating the role played by plg and activation of host fibrinolysis in alleviating staphylococcal infection with SAK-producing strains. Interestingly, plg and its endogenous activators, tPA and urokinase plg activator (uPA), have been shown to play a protective role in infections with various pathogens, such as *E. coli* [34, 35], *Pneumocystis carinii* [36], and *Cryptococcus neoformans* [37]. In addition, in the case of *S. aureus* infections, uPA [38, 39] and plg [40] have been showed to play a protective role. Our current findings underline the protective effects of the fibrinolytic system and demonstrate that bacterial activators of fibrinolysis might be as efficient in alleviating infection as endogenous plg activators. Given that the reactivity of SAK with plg from various mammalian species is highly variable [4], antivirulence properties of SAK might be evident only in infections of animals with SAK-sensitive plg (such as humans, dogs, or rabbits). The protective effect of the interaction between SAK and h-plg raises the possibility of using the SAK–h-plg system for therapeutic purposes in staphylococcal infections.

In summary, in this study we have shown that activation of plg by SAK reduced the severity of systemic staphylococcal infection.

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