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Staphylokinase Production by Clinical *Staphylococcus aureus* Strains

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Abstract

One of virulence factors produced by *Staphylococcus aureus* is staphylokinase (SAK), which enhances their proteolytic activity leading to tissue damage and improving bacterial invasiveness. In the present study we estimated the ability to produce staphylokinase by 95 *S. aureus* reference strains and clinical isolates from the airways of cystic fibrosis patients, from skin lesions and from infected bones. We would like to verify any relationship between SAK production and the types of clinical isolates as well as other biochemical properties and activities of these staphylococcal strains, which can be important for their pathogenicity. More than 62% of all tested strains were able to produce secreted type of SAK. Staphylokinase production was significantly more common in the isolates from skin and soft tissue infections than in any other group of tested staphylococci. The general tendencies in the selected properties or activities of both SAK(–) and SAK(+) isolates were similar. Our data confirm phenotypic dissimilarity in SAK production of *S. aureus* strains isolated from various types of infections. It is compatible with the biological role of staphylokinase and with hypothetical model of staphylokinase mediated bacterial invasion of host tissues. Thus, the estimation of SAK production by *S. aureus* isolates may be regarded as the parameter describing potential invasiveness of staphylococci and can be useful as a medical recommendation for the eradication of staphylococci carrier state.

Key words: *Staphylococcus aureus* clinical strains, staphylokinase (SAK), virulence

Introduction

Staphylococcus aureus is one of the common human pathogens. It permanently colonizes the epithelium of 20% of the population, transiently occurs in more than 60% (Foster, 2005). Thus *S. aureus* has a simple access to the host organisms and can occasionally cause both acute and chronic infections. These bacteria are responsible for a wide range of illnesses: from skin and soft tissue lesions like ulcers and furuncles, through food poisoning, to life threatening infections such as bacteremia followed by arthritis, osteomyelitis or endocarditis and septic shock (Foster, 2005; Krut *et al.*, 2003). There is no doubt that "invasive success" of *S. aureus* is associated with the broad spectrum of its virulence factors. This microorganism expresses a lot of secreted and cell-surface polysacchrides and proteins inclusive of many toxins and enzymes, which promote first the bacterial colonization, then the damage of host tissue, spreading of bacteria through organism, immune evasion and finally lead to fully-symptomatic disease (Cheung *et al.*, 2004; Foster, 2005; Heyer *et al.*, 2002; Otto *et al.*, 1999). One of these virulence factors is staphylokinase (SAK), also called fibrynolysin. SAK is 136-amino acid extracellular protein produced during the late exponential growth phase by *S. aureus* strains carrying the prophages which contain the *sak* gene (Bokarewa *et al.*, 2006; Jin *et al.*, 2004; Lähteenmäki *et al.*, 2001; Rooijakkers *et al.*, 2005). This protein is one of four human specific immune innate modulators, including chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylococcal complement inhibitor (SCIN) and enterotoxin A, which form a special cluster (called innate immune evasion cluster – IEC) on the conserved 3' end of β-hemolysin converting bacteriophages (van Wamel *et al.*, 2006). Staphylokinase, similarly to streptokinase secreted by some β-hemolytic group of streptococci or Pla – surface protease produced by *Yersinia pestis*, belongs to a group of bacterial plasminogen (PLG) activators (Lähteenmäki *et al.*, 2001; Rooijakkers *et al.*, 2005). PLG is the precursor of

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fibrinolytic protease: plasmin – an enzyme that degrades proteins of the extracellular matrix. SAK does not have an enzymatic activity itself but forms a 1:1 stoichiometric complexes with PLG, which convert other plasminogen molecules to plasmin (Jin *et al.*, 2004; Lähteenmäki *et al.*, 2001; Rooijakkers *et al.*, 2005). Thus staphylokinase enhances proteolytic activity of *S. aureus* strains against extracellular matrix proteins (ECM). Taking into consideration the fact that such ECM as collagen, laminin, fibronectin or elastin are the main components of tissue barriers and basement membranes, SAK can be regarded as a very important staphylococcal virulence factor, which leads to tissue damage and improves bacterial invasiveness (Bokarewa *et al.*, 2006; Lähteenmäki *et al.*, 2001).

In the present study we estimated the ability to produce soluble form of staphylokinase by 95 *S. aureus* strains selected from our collection. Most of these strains were clinical isolates representing different kinds of staphylococcal infections: from classic skin lesions like abscesses, ulcers or furuncles, through deep, difficult to treat infections of the bones, to specific, very often mixed airways infections of cystic fibrosis patients. Considering staphylokinase as a very important virulence factor of staphylococci, we would like to verify any relationship between SAK production and the types of clinical isolates. In our search we also included the group of reference *S. aureus* strains as a specific control group. Phenotypic features of these strains, along with their capability of SAK production, could not be modified by the contact with both host organism and other bacteria.

The gene coding SAK together with the genes for some other virulence factors form a special cluster IEC. In the light of this information and the fact, that staphylococci possess global regulatory systems of the genes (*e.g. agr*, *sar*), the dependence between different, apparently not connected features of these bacteria can be expected. Therefore, we decided to collate our knowledge about some characters and activities of the tested *S. aureus* strains, which can be important for their pathogenicity, with SAK production by these staphylococci. To this part of our search we chose the group of isolates from cystic fibrosis patients as the most representative, because of their numbers and the percentage of SAK(+) strains similar to those observed for natural and other clinical *S. aureus* populations.

Experimental

Materials and Methods

Characterization of *S. aureus* strains. Four groups of *S. aureus* strains were set up as: **(I)** laboratory reference strains of staphylococci (9 strains: Cowan 1 – overproducer of protein A, Reynolds – capsule serotype 5 prototype and Becker – capsule serotype 8 prototype, Wood 46 – overproducer of α-hemolysin, 8325-4 – *S. aureus* used to genetic manipulations, DU1090 – α-hemolysin negative mutant, MRSA 478 – MRSA class I prototype, MRSA 479 – MRSA class II prototype and MRSA 477 – MRSA class III prototype), **(II)** *S. aureus* isolated from airways of cystic fibrosis patients (59 strains) [from the Mother and Child Institute of Warsaw and from the Institute for Tuberculosis and Pulmonary Diseases, Rabka, Poland], **(III)** clinical strains isolated from skin lesions like abscesses, ulcers or furuncles (12 strains) [from the Clinic of Dermatology, Health Care Groupe, Łódź, Poland], **(IV)** clinical strains isolated from infected bones (15 strains) [from M. Copernicus' Hospital, Łódź, Poland]. All strains were subcultured on sheep blood agar to check their macro- and microscopic (by Gram staining) morphology and their hemolytic activity. Then the isolates were identified as *S. aureus* using conventional biochemical tests: detection of catalase (slide test with H_2O_2) and coagulase (tube test with rabbit plasma), decomposition of glucose and mannitol under aerobic and anaerobic conditions (tube test on Hugh-Leifson Medium), novobiocin susceptibility (disc diffusion test on Müller-Hinton Agar) and using latex agglutination assay, which detects clumping factor (CF). Strains were kept frozen (– 80°C) in Triptic Soy Broth (TSB; Difco, USA) with 15% of glycerol.

Some special features and activities of *S. aureus* strains isolated from the airways of cystic fibrosis patients were investigated in our previous studies (Sadowska *et al.*, 2000; Sadowska *et al.*, 2002). The type of capsular polysaccharide (CP) was estimated using monoclonal antibody against CP5 and CP8. Screening technique on Müeller-Hinton Agar with 6 μg/ml oxacillin and 4% NaCl was used to observe the profile of the resistance to methicillin. Also the ability to form small colony variant (SCV) was estimated after the passage of *S. aureus* strains in tryptic soy broth with 1 μg/ml of gentamicin.

Reagents. Human Glu-plasminogen was obtained from American Diagnostica (USA). This reagent was prepared from fresh human citrated plasma by lysine-Sepharose affinity chromatography in the presence of aprotinin. Recombinant SAK (rSAK) was purchased from ProSpec-Tany TechnoGene LTD (Israel) and the substrate for plasmin H-D-Val-Leu-Lys-pNA × 2HCl (S-2251) from Chromogenix (Italy). Todd-Hewitt Broth (THB), Todd-Hewitt Agar (THA) and sheep blood agar were obtained from BTL (Poland).

Culturing of *S. aureus* strains for SAK production. *S. aureus* strains were cultured on THA for 24 h at 37°C. One colony of each stahylococcal isolate was transferred into 2 ml of THB and incubated for 18 h at 37°C. The cultures were centrifuged (2600 × g, 10 min,

4°C) and the supernatants were collected. The supernatants (in duplicate) were tested for activity of soluble SAK and the selected bacteria for activity of surface-bound SAK.

SAK activity measurement in staphylococcal supernatants. Staphylokinase activity was determined by measuring plasmin's substrate hydrolysis in the presence of 1 μM plasminogen in Tris/HCl buffer (0.14 M NaCl, 1.5 M Tris/HCl, pH 7.2). Glu-plasminogen was incubated with culture supernatants for 1 h at 37°C to allow the conversion of plasminogen to plasmin. Plasmin formation was evaluated by hydrolysis of 4 mM chromogenic substrate S-2251 for 30 min at 37°C. The standard curve was performed using tenfold dilutions of rSAK (range: 0.078–5 μg/ml) preincubated with 1 μM plasminogen in Tris/HCl buffer. The absorbance reading for soluble form of SAK in bacterial supernatants and the standard curve of rSAK was measured at 405 nm on multifunction reader Victor 2 (Wallac, Finland). Mean absorbance values were converted on SAK concentration on the basis of the equation of trend line for the standard curve.

Determination of surface-bound SAK activity. The suspensions of staphylococci were prepared in 0.85% NaCl to a density equivalent to the McFarland turbidity standard of 3.0 by Densi-La-Meter (LaChema). Next, the bacteria were centrifuged (2600 × g, 10 min, 4°C), resuspended in THB and incubated for 4 h at 37°C with 1 μM Glu-plasminogen. The excess of plasminogen was removed by washing twice with 1 ml PBS. Finally, the bacteria were resuspended in Tris/HCl buffer (0.14 M NaCl, 1.5 M Tris/HCl, pH 7.2), transferred into 96-well plate and incubated with 4 mM substrate S-2251 for approximately 18 h at 37°C. Positive and negative controls were performed using *S. aureus* Cowan1 (SAK+) and *S. aureus* strain not-producing SAK, respectively. Both were prepared as 10^9 cfu/ml suspensions in Tris/HCl buffer with 4 mM plasmin substrate. The mean absorbance reading for surface-bound SAK of staphylococci was registered spectrophotometrically at 405 nm and then compared with the absorbance values obtained for the controls.

Statistical analysis. Chi-square test with Yates correction, Fisher test or V-square test were used to compare SAK production between all four groups of tested staphylococci and to correlate the ability to produce SAK with other properties of strains isolated from patients with cystic fibrosis. A $P < 0.05$ was considered significant.

Results

We examined the production of staphylokinase by 95 *S. aureus* strains divided into four groups: (I) laboratory reference strains of staphylococci (9 strains), (II) *S. aureus* isolated from airways of cystic fibrosis patients (59 strains), (III) clinical strains isolated from skin lesions (12 strains), (IV) clinical strains isolated from bones' infections (15 strains). Post-culture supernatant samples with SAK level below 0.3 μg/ml were considered as negative (–). SAK levels ranging from 0.3 to 2.5 μg/ml were assessed as low production and all samples with SAK level above 2.5 μg/ml as high production. The obtained results are presented in Table I as the percentage of *S. aureus* strains possessing or not the ability to produce soluble form of staphylokinase.

Table I
The percentage of *S. aureus* strains producing or not producing soluble form of staphylokinase (SAK)

Group No.	Type of *S. aureus* strains	SAK production (μg/ml)		
		negative (below 0.3)	low (0.3–2.5)	high (above 2.5)
I	lab reference (9 strains)	33%	22%	45%
II	cystic fibrosis (59 strains)	41%	42%	17%
III	skin lesions (12 strains)	8%	67%	25%
IV	infected bones (15 strains)	53%	14%	33%

More than 62% of all tested strains were able to produce soluble form of SAK: 67% strains from group I; 59% strains from group II; 92% strains from group III and 47% strains from group IV. It is noteworthy, that staphylokinase production was more common in isolates from skin and soft tissue infections (group III) than in any other group of *S. aureus* strains. However, statistically significant differences only between group III and IV were observed ($P = 0.04$). More than 22% of all SAK(+) strains secreted high amounts of soluble staphylokinase.

Almost 38% of all strains were classified as SAK non-producers, which was verified by a test performed for the detection of surface-bound staphylokinase. A few strains (representatives of all four groups) described as SAK(–) for a soluble form of this enzyme and *S. aureus* Cowan 1 as a positive control of soluble SAK producers were tested on cell-associated SAK. All examined strains, which did not secrete of soluble SAK, also did not possess surface-attached form of this enzyme.

The search for the correlation of soluble SAK production and some other properties of tested bacterial strains was performed for the isolates from airways of cystic fibrosis patients – the most numerous group (59 strains). The percentage of SAK(+) strains from this group was similar as for natural and other clinical

Table II
The soluble form of SAK production versus other properties and activities of *S. aureus* strains isolated from airways of cystic fibrosis patients

Properties/activities	Production of SAK released into the fluid phase	
	negative (24 strains)	positive (35 strains)
Type of capsule:		
– CP 5	4 (17%)	3 (9%)
– CP 8	12 (50%)	15 (43%)
– lack or other	8 (33%)	17 (49%)
Profile of resistance to methicillin (MRSA):		
– MSSA (susceptibility)	10 (42%)	19 (54%)
– MRSA class I	10 (42%)	7 (20%)
– MRSA class II	1 (4%)	0 (0%)
– MRSA class III	3 (13%)	9 (26%)
Ability to SCV formation	6 (25%)	7 (20%)

populations of staphylococci – about 60–70% (Bokarewa *et al.*, 2006; Jin *et al.*, 2003; van Wamel *et al.*, 2006). For this reason the isolates from airways of cystic fibrosis patients seem to be the most representative group for this kind of search. First of all we noticed, that hemolytic activity of both SAK(–) and SAK(+) staphylococci was similar (strong for most strains: 83% and 86% of SAK negative and positive strains, respectively). Little differences in the capability of anaerobic decomposition of mannitol between SAK(–) and SAK(+) *S. aureus* strains were observed (respectively 67% and 80% of strains were capable of mannitol fermentation), but the differences were not statistically significant ($P = 0.252$). Other correlated data are presented in Table II as the number and the percentage of *S. aureus* isolates possessing or not definite features or activity. Single results received for both SAK(–) and SAK(+) cystic fibrosis isolates differed insignificantly ($P > 0.05$) and general tendencies in their properties or activities were similar. Independently of the capability of SAK production, *S. aureus* strains possessed capsular polysaccharide type 8 (CP 8) more often than type 5 (CP 5), more than 40% of isolates were susceptible to methicillin. Also similar percentage of SAK(–) and SAK(+) strains SCV under gentamicin pressure was recovered.

Discussion

The pathogenicity of *S. aureus* is a complex process involving simultaneously many cell wall components and extracellular products and is very difficult to indicate the importance of their single virulence factor. The observations of the effects of SAK production by *S. aureus* and the conclusions drawn sometimes seem to be contradictory. SAK-deficient *S. aureus* isolates happened to be described as more dangerous causing the lethal bacteremia more frequently than staphylococci producing SAK. Moreover, the production of staphylokinase by nasal isolates as one of the adaptive mechanisms of *S. aureus* symbiosis with the host was suggested (Bokarewa *et al.*, 2006; Jin *et al.*, 2003). On the other hand, it was proved that staphylococcal strains producing SAK were protected against the bactericidal effect of human α-defensins (HNP-1, HNP-2) and against opsonization by both immunoglobulin G and C3b/C3bi, which could promote the invasion of host tissues by these strains (Jin *et al.*, 2004; Rooijakkers *et al.*, 2005; van Wamel *et al.*, 2006). Such various roles of SAK seem to be dependent on the stage of infection or current needs of staphylococci. It can be presumed, that at the beginning of infection SAK production should be inhibited to prevent the proteolysis of ECM being very important for bacterial adhesion. Then, during the invasion of host tissue, the expression of SAK should be increased allowing degradation of the junctions between host cells or destruction of basement membranes. Based on these considerations, we decided to estimate the ability to the secretion of staphylokinase by 95 *S. aureus* strains and find the relationships between its production and the types of infection or other biochemical properties and activities of these strains.

It was demonstrated that more than 62% of all our tested *S. aureus* strains were able to produce and release staphylokinase. Van Wamel *et al.* (2006) also discovered the different IEC types containing *sak* gene in 76.6% of clinical isolates of staphylococci. The carrying of such genetic mobile elements coding the virulence factors being able to affect human innate immune system (*e.g.* SEA modulates the functions of chemokine receptors, SAK and SCIN possess anti-opsonic capacity, CHIPS blocks chemotaxis) is very profitable for bacteria (Jarraud *et al.*, 2002; van Wamel *et al.*, 2006). Thereby, the selective distribution of some genes, for instance these coding the superantigens, among *S. aureus* clinical strains was also described by Ferry *et al.* (2005), Omoe *et al.* (2005) and van Belkum *et al.* (2006). This phenomenon has probably developed during evolutional adaptation of bacteria to the specific micro-environmental conditions appearing *in vivo* in different kind of infections or even during their stages. On the other hand, taking to consideration our results for control group of staphylococci – group I (almost 70% of these strains were SAK+), the ability to staphylokinase production seems to be profitable for these bacteria, even if they don’t have contact with host organism and this feature can not be created by special environmental conditions.

Distribution of SAK production was also described by Jin *et al.* (2003), whose observations were similar to our results. We noticed significantly more frequent staphylokinase production in isolates from skin and soft tissue infections (group III) than in any other group of tested *S. aureus* strains. They observed that SAK positive strains were less common (1.7 times) among the isolates from patients with lethal bacteremia than among nasal carriage isolates. It confirms the earlier observations of unexpected lack of SAK production in staphylococci invading internal organs in comparison with these colonizing mucosal tissue and registered SAK production in almost all staphylococcal isolates obtained from skin and mucosa (Bokarewa *et al.*, 2006; Jin *et al.*, 2003). Our results are compatible with one of the models of staphylokinase mediated bacterial invasion. It is suggested, that SAK-PLG complexes may help staphylococci cleave the infectious focus or abscess from the fibrin net, thus enabling these bacteria to enter into the deeper host tissue (Bokarewa *et al.*, 2006). This is a good explanation for our observations, that staphylokinase was produced mainly by strains isolated from skin lesions like abscesses, ulcers or furuncles.

In the light of the information about special cluster IEC coding *sak* gene together with the genes for some other virulence factors and of the fact, that staphylococci possess global regulatory systems of the genes (*e.g. agr*, *sar*), the dependence between different features of these bacteria can be expected. Therefore we decided to correlate some other properties and activities of tested *S. aureus* strains with their ability to produce SAK. In this part of our research, we chose the group of isolates from cystic fibrosis patients as the most representative, because of their number and the percentage of SAK(+) strains similar to those observed for natural and other clinical *S. aureus* populations. During the collection of the strains and the preparation of their stocks we checked the hemolytic activity of these bacteria. It was proved that the ability of SAK(+) and SAK(–) strains to produce hemolysin was similar. This observation is interesting with regard to the known effect of inactivation of β-hemolysin gene by the insertion of staphylokinase-carrying bacteriophage to the bacterial genome (Bokarewa *et al.*, 2006; Jin *et al.*, 2003; Lähteenmäki *et al.*, 2001). Although that α-hemolysin is mainly responsible for hemolytic activity of *S. aureus* strains. In our previous studies (Sadowska *et al.*, 2000; Sadowska *et al.*, 2002) we also estimated some special features and the activity of these *S. aureus* strains from group II. Now, we noticed that the production of soluble SAK did not correlate with such staphylococci features as type of polysaccharide capsule ($P = 0.4292$), the profile of resistance to methicillin ($P = 0.3409$) or small colony variant (SCV) formation under antibiotic pressure ($P = 0.6518$). Therefore SAK production seems not to have any importance for the strains invading the lungs in cystic fibrosis patients.

In conclusion, our data demonstrate that phenotypic differences in secreted SAK production exist among *S. aureus* strains isolated from various kinds of infections. It is compatible with the biological role of staphylokinase and the theoretical model of staphylokinase mediated bacterial invasion of host tissues. Thus, the simple laboratory method for the estimation of SAK production by *S. aureus* isolates (*e.g.* strains isolated from the carriers) may be accepted as the parameter describing potential invasiveness of staphylococci. Such knowledge can be useful as a medical recommendation to eradication of staphylococci carrier state in particular cases.

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