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; Heyer et al., 2002; Otto et al., 1999). One of these virulence factors 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
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; Rootiakkers 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 H2O2) 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 Müller-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 staphylococcal 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 Cowan 1 as a positive control of SAK(+) and S. aureus strain not-producing SAK, respectively. Both were prepared as 10^6 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.

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-associated 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

<table>
<thead>
<tr>
<th>Properties/activities</th>
<th>Production of SAK released into the fluid phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>negative (24 strains)</td>
</tr>
<tr>
<td>Type of capsule:</td>
<td></td>
</tr>
<tr>
<td>– CP 5</td>
<td>4 (17%)</td>
</tr>
<tr>
<td>– CP 8</td>
<td>12 (50%)</td>
</tr>
<tr>
<td>– lack or other</td>
<td>8 (33%)</td>
</tr>
<tr>
<td>Profile of resistance to methicillin (MRSA):</td>
<td></td>
</tr>
<tr>
<td>– MSSA (susceptibility)</td>
<td>10 (42%)</td>
</tr>
<tr>
<td>– MRSA class I</td>
<td>10 (42%)</td>
</tr>
<tr>
<td>– MRSA class II</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>– MRSA class III</td>
<td>3 (13%)</td>
</tr>
<tr>
<td>Ability to SCV formation</td>
<td>6 (25%)</td>
</tr>
</tbody>
</table>

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 statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252). Other correlated data are presented in Table II as the number and the statistically significant (P = 0.252).

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 function 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 evolutionary 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.

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 function 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 evolutionary 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 abscesses 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 staphylococcal 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.

Literature


