The human fibrinolytic system is a target for the staphylococcal metalloprotease aureolysin

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The major opportunistic pathogen Staphylococcus aureus utilizes the human fibrinolytic system for invasion and spread via plasminogen binding and non-proteolytic activation. Because S. aureus secretes several proteases recently proposed as virulence factors, we explored whether these enzymes could add to the activation of the host’s fibrinolytic system. Exposure of human pro-urokinase [pro-uPA (where uPA is urokinase-type plasminogen activator)] to conditioned growth media from staphylococcal reference strains results in an EDTA-sensitive conversion of the plasmin(ogen) to conditioned growth media from staphylococcal urokinase [pro-uPA (where uPA is urokinase-type plasminogen activator)], the major PAI (plasminogen activator) of plasminogen activator SAK (staphylokinase) [7–11]. S. aureus is an opportunistic human pathogen that colonizes numerous tissues and organs and can lead to a variety of benign to severe and often fatal disorders, including local and systemic infections, as well as toxic syndromes [12,13]. Moreover, this bacterium represents the worldwide leading cause of nosocomial infections and has an alarming capacity to develop resistance to antibiotics, demanding for a better knowledge of its pathobiology [14,15].

Among the array of secreted staphylococcal factors, a number of proteases, including the serine protease V8 (SspA/V8) and SplA–SplF, the cysteine proteases ScpA (staphopain A) and SspB (staphopain B) and the metalloprotease aureolysin, have recently attracted attention as potential virulence factors [16]. Aureolysin is a neutral, calcium-stabilized, zinc-dependant metalloprotease and belongs to the superfamily of M4, or thermolysin-like, endopeptidases [17]. Most, if not all, staphylococcal strains display a functional gene encoding aureolysin [18], which is synthesized and secreted throughout the bacterium represents the worldwide leading cause of nosocomial infections and has an alarming capacity to develop resistance to antibiotics, demanding for a better knowledge of its pathobiology [14,15].

INTRODUCTION

The plasminogen activation system comprises the serine protease zymogen plasminogen, its two proteolytic activators uPA (urokinase-type plasminogen activator) and tPA (tissue plasminogen activator), the major PAI (plasminogen activator inhibitor) (PAI-1) and the plasin inhibitor α2-AP (α2-antiplasmin) [1,2]. Once activated, plasmin displays a broad-spectrum trypsin-like enzymatic activity towards a number of substrates including fibrin, extracellular matrix components, basement membrane proteins and MMPs (matrix metalloproteinases). Significantly, plasmin also targets and activates its own activators, uPA and tPA, thus providing the system with a remarkable capacity for auto-amplification. The fibrinolytic system is involved in a variety of (patho)physiological processes including reproduction, embryogenesis, vascular homeostasis, inflammation, wound healing, as well as malignancies [2–4].

A large body of evidence indicates that some bacteria are capable of utilizing this otherwise tightly regulated proteolytic system, thereby promoting their spread and invasion, both through overexpression of its components, surface expression of plasminogen-binding sites, activation of the zymogens and/or inactivation of the protease inhibitors [5,6]. Among these micro-organisms, a remarkable example is represented by the Gram-positive bacterium Staphylococcus aureus, which enhances uPA production by mammalian cells, expresses lysine-rich binding sites for plasminogen and secretes the non-proteolytic plasminogen activator SAK (staphylokinase) [7–11]. S. aureus is an opportunistic human pathogen that colonizes numerous tissues and organs and can lead to a variety of benign to severe and often fatal disorders, including local and systemic infections, as well as toxic syndromes [12,13]. Moreover, this bacterium represents the worldwide leading cause of nosocomial infections and has an alarming capacity to develop resistance to antibiotics, demanding for a better knowledge of its pathobiology [14,15].

Key words: aureolysin, infection, proteolysis, Staphylococcus aureus, staphylokinase, urokinase.
antimicrobial peptide, LL-37, and (iii) the serpin-type plasma protease inhibitors $\alpha_1$-antichymotrypsin and $\alpha_1$-proteinase inhibitor, thereby potentially promoting bacterial escape from the host immune system, as well as uncontrolled proteolysis, leading to host tissue destruction [16,23]. Altogether, it is thus tempting to speculate that aureolysin might display key functions during host colonization, infection and invasion.

We now report that *S. aureus* reference and clinical strains are capable of converting thezymogen pro-uPA into its active form, a feature mediated by aureolysin. Interestingly, we demonstrate that aureolysin also targets other components of the plasminogen activation system, including plasminogen, PAI-1 and $\alpha_2$-AP. We thus propose that the activation of the host’s fibrinolytic system by this metalloprotease might be an alternative mechanism to SAK expression, contributing to bacterial spread and invasion.

**EXPERIMENTAL**

**Materials**

Human Glu-plasminogen and plasmin, purified from plasma, and human two-chain active urokinase [uPA; 100000 IU (international units)/mg], purified from urine, were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.), Chromogenix/Haemochrom Diagnostica (Esen, Germany) and ProSpec-Tany TechnoGene (Rehovot, Israel) respectively. Recombinant human pro-uPA, expressed in *Escherichia coli*, was a gift from A.G. Saunders (Grüentalh, Aachen, Germany). Recombinant human PAI-1, expressed in *E. coli*, was produced and purified as previously reported [24]. Human $\alpha_2$-AP, purified from plasma, was a gift from J.J. Enghild (University of Aarhus, Aarhus, Denmark [25]). Aureolysin was purified from conditioned growth medium of *S. aureus* strain V8-BC10 by the procedure initially described by Drapeau [26]. The enzyme purity was determined by SDS/PAGE, and the protein concentration was determined using a BCA (bicinchoninic acid) kit (Sigma–Aldrich), with BSA (Sigma–Aldrich) as a standard. Aureolysin, which was found to be in its active form, as judged by $\alpha_1$-macroglobulin titration, was stored at $-20^\circ$C as a 0.7 mg/ml (i.e. 21 µM) stock solution. Protease inhibitors were obtained from Sigma–Aldrich.

The chromogenic plasmin substrate B-Val-Leu-Lys-pNA (where pNA is p-nitroanilide) (DVLK-902) was obtained from Molecular Innovations (Southfield, MI, U.S.A.), the chromogenic uPA substrates Tos-Gly-Pro-Arg-pNA (L-1480) and Bz-β-Ala-Gly-Arg-pNA (where Bz is benzoyl) (Pefachrome uPA) were from Bachem (Bubendorf, Switzerland) and Loxo (Dossenheim, Germany) respectively, and the fluorogenic uPA substrate Tos-Gly-Pro-Arg-pNA (L-1480) and Bz-Leu-Gly-Arg-AMC (where Boc is t-butoxycarbonyl and AMC is 7-amino-4-methylcoumarin) was from Bachem. A rabbit pAb (polyclonal antibody) directed to human plasminogen was obtained from Dako (Glostrup, Denmark). A chicken pAb directed to human uPA and strongly reacting with the A-chain was a gift from N. Grebenchtchikov (University Medical Center Nijmegen, Nijmegen, The Netherlands [27]). HRP (horseradish peroxidase)-conjugated antibodies against rabbit or chicken IgG were from Dianova (Hamburg, Germany) and Sigma–Aldrich respectively.

**Bacterial strains and collection of conditioned growth media**

The following staphylococcal bacterial strains were used: Newman (NTCC 8178); 8325-4 (NTCC 8325); 8325-4 aur−, corresponding to the strain 8325-4 with an isogene knockout of the gene encoding aureolysin [20]; V8-BC10 [26]; a collection of nine clinical isolates (i.e. strain numbers 1, 12, 38, 46, 59, 68, 100, 101 and 103).

For collection of conditioned growth media [CM (conditioned medium)], bacteria were grown in TSB medium (tryptisose soy broth medium; Sigma–Aldrich) for 10 h at 37°C with shaking until cells had reached the stationary growth phase. Bacterial cultures were then centrifuged at 14 000 g for 20 min at 4°C, and the supernatants were collected and stored at $-20^\circ$C before further use.

**PCR analysis**

Bacteria were lysed in a buffer containing 20 mM Tris, 2% (v/v) Triton X-100 and 2 mM EDTA (pH 8.0), in the presence of 2.5 units of lysostaphin (Sigma–Aldrich), for 30 min at 37°C before genomic DNA extraction using the Genomic Mini kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer’s instructions. PCR amplification was performed using 0.5 unit of Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and the following primers: 5'-GTGAGGAATTTTCAAGATGC-3', 5'-CCACGCTACTTTATCCATGC-3' (forward and reverse primers for aureolysin gene detection respectively; IBB PAN, Warsaw, Poland), resulting in a 321 bp PCR product. The strains 8325-4 and 8325-4 aur− were used as positive and negative controls respectively.

**Aureolysin gelatin zymography**

Detection of aureolysin-related proteolytic activity was performed as reported previously [20]. Briefly, purified aureolysin (50 ng) or CM from clinical strains (10 µl) was treated for 1.5 h at 37°C with 5% (w/v) SDS, prior to SDS/PAGE on 12% gels containing 0.2% (w/v) gelatin. After electrophoresis, gels were washed twice with 2.5% Triton X-100 and developed overnight in a 0.2 M Tris (pH 7.8) buffer containing 5 mM CaCl₂ and 2 mM of the V8 protease inhibitor 3,4-dichloroisocoumarin. Alternatively, the metalloprotease-related proteolytic activity was neutralized in the samples and in the development buffer by the addition of 10 mM EDTA instead of CaCl₂. Gels were finally stained with Amido Black.

**Exposure of pro-uPA or plasminogen to CM**

Pro-uPA or plasminogen was adjusted to 200 µg/ml in CM diluted 1:2 (v/v) in a 100 mM Tris and 100 mM NaCl (pH 7.5) buffer (Tris/NaCl buffer), and reaction continued for 5 h at 37°C. Alternatively, incubation was carried out in the presence of protease inhibitors as indicated in the legend to Figure 1. Samples were frozen before preparation for SDS/PAGE or proteolytic activity measurement.

**Exposure of pro-uPA, plasminogen, PAI-1 or $\alpha_2$-AP to purified aureolysin**

Pro-uPA, plasminogen, PAI-1 or $\alpha_2$-AP, adjusted to 20 µg/ml in a Tris/NaCl buffer containing 5 mM CaCl₂ (Tris/NaCl/CaCl₂ buffer), were either mixed with aureolysin in the range 2.25–45 nM [i.e. E/S (enzyme/substrate) molar ratios varying from 1:100 to 1:8] for 1, 5 or 24 h at 37°C or left untreated as a control. To inhibit aureolysin proteolytic activity, CaCl₂ was omitted and replaced by 5 mM EDTA in the reaction mixture. Samples were then frozen before preparation for SDS/PAGE or proteolytic activity measurement.

For experiments performed in plasma-derived buffer, human plasma from a healthy volunteer was collected according to standard procedures and diluted to 5% in a Tris/NaCl/CaCl₂...
The activities of CM on L-1480 were found to be insignificant (and 100% activation of the zymogen present in the assay respectively. The intrinsic proteolytic to TSB medium left the zymogen intact and inactive (µcontrol, is represented as a dotted (0.2 independent experiments. The proteolytic activity of purified uPA, used as an external positive

Exposure of pro-uPA to aureolysin was then performed exactly to prevent pro-uPA activation by traces of active plasmin.

Fig. 1 A secreted staphylococcal metalloprotease activates pro-uPA

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buffer containing 10 µM of the plasmin inhibitor aprotinin in order to prevent pro-uPA activation by traces of active plasmin. Exposure of pro-uPA to aureolysin was then performed exactly as described above.

Activity measurement of human proteases

Samples were placed in a 96-well plate and adjusted to a 160 µl final volume in a 100 mM Tris, 0.05% Tween 20 and 0.01% BSA (pH 7.5) buffer (for DVLK-902 and L-1480) or in a 50 mM Tris and 150 mM NaCl (pH 8.0) buffer (for Pefachrome) in the presence of 0.19 mM DVLK-902 or 0.13 mM L-1480 or Pefachrome. The release of pNA at 37 °C was then monitored at 405 nm with a spectrophotometer (SLT-Labinstruments, Grödig, Austria) over a 30 min time period.

To assay the inhibitory activity of aureolysin-treated PAI-1 or α2-AP, the serpins were allowed to interact with their target proteases uPA (for PAI-1) or plasmin (for α2-AP) for 30 min at room temperature (20°C) before measurement of the remaining proteolytic activity of the mixture.

Determination of the kinetic parameters of pro-uPA activation

Aureolysin (1 nM) or plasmin (0.01 nM), which had been standardized by active-site titration using p-nitrophenyl-β-guanidinobenzoate [28], were incubated with varying concentrations of pro-uPA (0–10 µM) in a 50 mM Hepes, 150 mM NaCl, 0.01 % azide and 0.01 % Triton X-100 (pH 7.4) buffer. Zymogen activation was followed by recording the release of AMC generated by cleavage of the reporter substrate Boc-Leu-Gly-Arg-AMC (20 µM) by activated uPA. In each experiment, the specific activity of uPA towards the reporter substrate was determined using active-site-titrated uPA (0–10 nM) under the same conditions; cleavage by pro-uPA (or contaminating uPA), aureolysin and plasmin was negligible. Results were recorded every 30 s for 30 min using a HTS 7000 Bio Assay Reader (PerkinElmer, Rodgau-Jügesheim, Germany), with λexcitation of 360 and 465 nm respectively.

The time-dependent increase in AMC was fitted to a modification of an integrated rate equation describing a coupled zymogen activation and reporter reaction initially used to analyse the activation of plasminogen [29,30]:

\[
AMC(t) = 0.5a \cdot V(t - t_0)^2 + AMC_0,
\]

where AMC(t) and AMC0 are the concentrations of AMC measured at time t and baseline respectively, t0 the delay between the start of the reaction and the first measurement, V the velocity of zymogen activation and a, the specific activity of uPA towards the reporter substrate. The zymogen activation velocities V were at different pro-uPA concentrations calculated from two different experiments were subsequently fitted to the Michaelis–Menten equation to obtain the kinetic parameters Km, Vmax, and kcat/Km.

Both curve-fitting steps were performed with proFit (Quantum Soft, Uetikon am See, Switzerland) using a Levenberg–Marquardt algorithm.

Protein chemical analysis and immunoblotting

Proteins (500 ng per well for silver staining or 100, 200 or 2000 ng per well for immunoblotting) were reduced in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol for 5 min at 95°C, followed by SDS/PAGE. Separated proteins were then either silver-stained according to standard procedures, or transferred on to PVDF (Pall, Dreieich, Germany) membranes prior to immunoblotting. After blocking with non-fat skimmed milk, membranes were probed with the primary antibody, as indicated in the Figure legends, and bound antibodies were detected by an HRP-coupled secondary antibody (dilution: 1:10000) followed by a chemiluminescent reaction (ECL®, Amersham Biosciences, Little Chalfont, Bucks., U.K.).

For N-terminal microsequencing, pro-uPA, plasminogen or α2-AP were adjusted to 400 µg/ml in a Tris/NaCl/CaCl2 buffer and exposed to aureolysin in an E/S molar ratio of 1:100, or left untreated as a control, for 1–2 h at 37°C. Proteins were then solubilized and reduced as described above before SDS/PAGE separation on 12% gels (or 16.5% Tris/Tricine gels for

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α₂-AP-related low-molecular-mass fragment) prior to transfer on to PVDF membranes. Protein bands visualized by Coomassie Brilliant Blue staining were finally excised and subjected to Edman degradation (pulse liquid-phase sequencer, Procise 492, Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS

Activation of human pro-uPA by a secreted staphylococcal metalloprotease

To study the interactions of extracellular proteases secreted by S. aureus with the human fibrinolytic system, we first exposed plasminogen and pro-uPA to CM from staphylococcal reference strains, and subsequently analysed the reaction mixture by immunoblotting and protease activity measurement.

Exposure of plasminogen (molecular mass ≈ 97 kDa) to CM from the strains 8325-4 and V8-BC10 (the latter strain being well characterized for its high protease expression levels [26]) for 5 h resulted in the processing of the zymogen into several molecular species, which were clearly distinct from those generated by exposure of plasminogen to its activator uPA (results not shown). Protease activity measurement confirmed the absence of detectable plasminogen activation by the bacterial secreted factors (results not shown). Conversely, a 5 h exposure of pro-uPA (molecular mass ≈ 49 kDa) to CM from the above-mentioned strains resulted in the processing of the zymogen into two major ~28 and 20 kDa molecular species, which were similar to the B- and A-chains generated by exposure of pro-uPA to its activator plasmin (Figures 1A and 1C). While processing of pro-uPA was incomplete when the zymogen was exposed to CM from strain 8325-4, it was nearly complete after exposure to CM from strain V8-BC10. This latter observation was also confirmed by protease activity measurement (Figures 1B and 1D).

To identify the secreted staphylococcal enzyme(s) responsible for pro-uPA activation, we next exposed pro-uPA to CM in the presence of an array of protease inhibitors (Figure 1). Western blotting and protease activity measurement indicated that, in contrast with inhibitors of serine, aspartate and cytosine proteases, a cocktail of protease inhibitors as well as EDTA efficiently inhibited pro-uPA activation by bacterial CM (Figures 1A–1D). Among the proteolytic enzymes secreted by S. aureus, aureolysin is the only known metalloprotease [16,20]; therefore, this neutral thermolysin-like protease was the candidate to cause the EDTA-sensitive proteolytic activation of pro-uPA by staphylococcal CM. To investigate this hypothesis, we next collected CM from a strain deficient in the gene encoding aureolysin (i.e. 8325-4-aar) [20] and used it for incubation with pro-uPA. In marked contrast with that from the parental 8325-4 strain, CM from this aureolysin-deficient strain was indeed unable to activate pro-uPA (Figures 1E and 1F).

Proteolytic activation of human pro-uPA by purified aureolysin

To prove further that aureolysin is the pro-uPA-activating enzyme secreted by S. aureus, we exposed pro-uPA to concentrations of purified aureolysin in the range 4.5–45 nM (i.e. E/S molar ratios from 1:100 to 1:10). Incubation for 1–24 h prior to SDS/PAGE of the reaction mixture revealed a conversion of the zymogen into a major ~28 kDa species and into minor ~20 kDa products (Figure 2A). Processing of pro-uPA, which was already detectable after a 1 h exposure to a protease concentration as low as 4.5 nM, was time- and concentration-dependent and was completely inhibited in the presence of EDTA. As illustrated in Figure 2(B), N-terminal microsequencing of the 28 kDa fragment, which remained stable and accumulated over time, demonstrated that it is generated by hydrolysis of the Lys158–Ile159 peptide bond and thus corresponds to the B-chain of uPA. The lower-molecular-mass species were identified as degradation products of the A-chain, resulting from cleavage between the growth factor and kringle domains and from further cleavage within the former domain. Exposure of pro-uPA to various concentrations of aureolysin for different periods of time followed by protease activity measurement confirmed the activation of the zymogen by the bacterial metalloprotease, occurring immediately after 1 h of exposure to 4.5 nM aureolysin and reaching a maximum after 5 h of exposure to 45 nM enzyme (Figure 2C). It is important to note that, when assayed under similar E/S molar ratios, none of the other purified staphylococcal proteases tested, including the serine proteases V8, SpLA and SpIB, as well as the cysteine proteases ScpA and SpSB, were able to activate pro-uPA (results not shown).

To characterize the efficiency of pro-uPA activation by aureolysin, we subsequently determined the kinetic parameters (Figure 2D), which were as follows: $k_{cat} = 0.3 ± 0.1 \text{s}^{-1}$, $K_m = 100 ± 28 \text{µM}$ (extrapolated) and $k_{cat}/K_m = 2.6 ± 0.2 \times 10^{-5} \text{M}^{-1} \cdot \text{s}^{-1}$, whereas the catalytic efficiency of human plasmin to activate pro-uPA, determined in parallel under identical conditions, was approx. 300-fold higher ($k_{cat} = 2.0 ± 0.03 \text{s}^{-1}$, $K_m = 2.3 ± 0.1 \text{µM}$ and $k_{cat}/K_m = 7.7 ± 0.2 \times 10^{-3} \text{M}^{-1} \cdot \text{s}^{-1}$).

Finally, to mimic S. aureus-induced vascular leakage, we exposed pro-uPA to 4.5–45 nM aureolysin in the presence of 5% human plasma for 5 h. Western-blot analysis of the resulting proteins again revealed an EDTA-sensitive conversion of the pro-enzyme into its A- and B-chains (Figure 2E). Along with this, an increased proteolytic activity of the reaction mixture towards an uPA-specific chromogenic substrate was detectable for 45 nM aureolysin (Figure 2F), although pro-uPA activation was clearly less efficient than that observed in the absence of plasma (compare Figures 2A and 2C with Figures 2E and 2F). This probably reflects the presence of protease inhibitors, including the potent aureolysin inhibitor α₂-macroglobulin, and of competitive aureolysin substrates [16,31–34], in normal human plasma.

pro-uPA activation by clinical staphylococcal isolates

We next explored the relevance of the aureolysin-dependent pro-uPA activation using CM from clinical S. aureus isolates. For this, clinical strains were collected from an asymptomatic nasal carrier and from eight patients suffering from various local and systemic staphylococcal infections representative for the variety of tissues that are colonized or infected by S. aureus (Table 1). PCR analysis of these strains indicated that all the nine strains were positive for the gene encoding aureolysin.

Pro-uPA was exposed for 5 h to CM from these strains before Western-blot analysis and protease activity measurement were performed (Figures 3A and 3B). A marked (i.e. in the range of 50%) conversion of pro-uPA into its active form was noted with CM from one of the clinical isolates (i.e. strain number 1), and a smaller but significant (i.e. at least 10%) activation of the zymogen was also observed with CM from five other strains (i.e. strain numbers 12, 48, 59, 68 and 100). Significantly, pro-uPA activation was completely inhibited in the presence of EDTA, suggesting a predominant participation of aureolysin. To strengthen this assumption, we assayed the CM from these strains for aureolysin-related proteolytic activity. As depicted in Figure 3(C) and in Table 1, gelatin zymography experiments
The proteolytic activity of purified uPA is represented as a dotted (0.2 activity of Aur was blocked with 5 mM EDTA added to the reaction mixture. (E)

activity was measured by spectrophotometry using the uPA-specific chromogenic substrate side indicate the location of pro-uPA and of the A- and B-chains of uPA. (F)

µm aprotinin in order to disable traces of active Pm. Alternatively, the proteolytic staining. The depicted panel is representative of independent experiments, and arrows on the left-hand side indicate the location of pro-uPA and of the A- and B-chains of uPA. (A) Proteins were subjected to SDS/PAGE under reducing conditions followed by silver staining. The depicted panel is representative of independent experiments, and arrows on the left-hand side indicate the location of pro-uPA and of the A- and B-chains of uPA. (B) Peptide bonds targeted by Aur within pro-uPA were determined by N-terminal microsequencing (GFD, growth factor domain; K, kringle domain; C, catalytic domain). Dots indicate the location of the amino acid residues constituting the serine protease catalytic triad, while arrows indicate the position of cleavage sites. (C) uPA proteolytic activity was measured using the L-1480 chromogenic substrate, which was found not to be cleaved by Aur (results not shown). Black (E/S=1:100) and grey (E/S=1:10) histograms represent means ± S.E.M. for three independent experiments.

Hydrolysis of plasminogen, PAI-1 and α2-AP by purified aureolysin

To determine whether aureolysin also targets other components of the plasminogen activation system, we next assessed its impact both on the zymogen plasminogen and on the regulatory serpins PAI-1 and α2-AP.

Exposure of plasminogen to aureolysin concentrations in the range 2.25–22.5 nM (i.e. E/S molar ratios from 1:100 to 1:10) for 1–24 h resulted in an EDTA-sensitive conversion of the zymogen into truncated molecular species of ∼67.5, 61 and 39.5 kDa, as evaluated by SDS/PAGE analysis (Figure 4A). According to their molecular mass and N-terminal sequences (Figures 4A and 4D respectively), these fragments correspond to previously reported carbohydrate variants of angiostatin, encompassing the kringle domains 1–4 (molecular mass ∼67.5, 61 and 39.5 kDa, N-terminus Lys-Val-Tyr-Leu/Val-Tyr-Leu-Ser) [35] and to mini-plasminogen, encompassing the kringle domain 5 and the catalytic domain of the serine protease (molecular mass ∼39.5 kDa, N-terminus Val-Val-Ala-Pro). According to protease activity measurement, aureolysin was indeed unable to activate plasminogen into plasin (in fact, none of the other purified staphylococcal proteases mentioned above, including V8, SpLA, SpIB, ScPA and SspB, was able to do so; results not shown). It is of note that aureolysin did not destroy the zymogen, as the cleavage products could subsequently be activated by uPA, either purified, or as generated via exposure of pro-uPA to the bacterial enzyme (results not shown). Moreover, aureolysin-generated mini-plasmin, although devoid of its lysine-binding sites, was still endowed with fibrinolytic capacities, as evaluated on a fibrin plate (results not shown).

Exposure of PAI-1 to aureolysin at similar E/S molar ratios and time periods resulted in degradation of the inhibitor (Figure 4B). To assess the impact of aureolysin–PAI-1 interaction on the line and corresponds to 10 and 100 % activation of the zymogen present in the assay, respectively. The intrinsic proteolytic activity of plasma-derived buffer on Pelachrome was found to be insignificant (F), and exposure of pro-uPA to aprotinin-containing, plasma-derived buffer left the zymogen intact and inactive (E, F).

Table 1 Origin and characteristics of the staphylococcal clinical strains assayed for their capacity to activate human pro-uPA

Table 1. Origin and characteristics of the staphylococcal clinical strains assayed for their capacity to activate human pro-uPA

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Origin</th>
<th>aur gene</th>
<th>Aureolysin-related proteolytic activity</th>
<th>Capacity to activate pro-uPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pneumonia</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Nasal swabs</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>38</td>
<td>Skin infection</td>
<td>+</td>
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<td>46</td>
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<td>68</td>
<td>Wound infection</td>
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<td>100</td>
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<td>Sepsis</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>103</td>
<td>Sepsis</td>
<td>+</td>
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</table>

*Clinical strains were tested for the presence of the aur gene, encoding aureolysin, as described in the Experimental section. +’ refers to the presence of the 321 bp PCR product corresponding to the aur gene.

†Aureolysin-related proteolytic activity displayed by CM was assayed by gelatin zymography as depicted in Figure 3. ‘+’ and ‘–’ refer to strong, medium and weak to no gelatinolytic activity respectively.

‡The capacity of CM for activating human pro-uPA was tested through Western blotting and protease activity measurement as depicted in Figure 3. ‘+’, ‘++’ and ‘–’ refer to strong, medium and weak to non-detectable uPA activations respectively.

indeed confirmed that aureolysin activity present in these CM correlates with their capacity to activate pro-uPA.
Nine staphylococcal clinical strains (numbers 1, 12, 38, 59, 68, 100, 101 and 103) were cultivated to the stationary growth phase before collection of CM. Pro-uPA (200 µg/ml) was exposed either to CM diluted 1:2 or to TSB medium in the presence or absence of plasmin (Pm) (4 µg/ml; E/S = 1:100), for 5 h at 37°C. Alternatively, the proteolytic activity of metalloproteases was neutralized by addition of 5 mM EDTA in the reaction mixture. 

(A) Proteins were subjected to SDS/PAGE under reducing conditions followed by immunoblotting using a chicken anti-uPA pAb (dilution 1:2000). Panels included are representative of independent experiments, and arrows on the left-hand side indicate the location of pro-uPA and of the A- and B-chains of uPA. 

(B) uPA proteolytic activity was measured by spectrophotometry using the chromogenic substrate L-1480. Histograms represent the means ± S.E.M. for three independent experiments. The proteolytic activity of purified uPA is represented as a dotted (0.2 µg) and a bold (2 µg) line, which correspond to 10 and 100 % activation of the zymogen present in the assay respectively. The intrinsic proteolytic activity of CM on L-1480 was found to be insignificant (results not shown). 

(C) The proteolytic activity of purified aureolysin (Aur; 50 ng) and CM from the clinical isolates (20 µl) was visualized using gelatin zymography in the presence of 5 mM CaCl2 and 2 mM of the V8 protease inhibitor 3,4-dichloroisocoumarin. The panel included is representative of independent assays. The arrow on the left-hand side indicates the location of Aur-related gelatinolytic areas, whereas asterisks indicate the location of Aur-related shifted areas, which were both determined to be EDTA-sensitive (results not shown).
Aureolysin activates the fibrinolytic system

Ph37 and Lys158–Ile159), plasminogen (i.e. Lys72–Lys78, Lys78–Val79 and Ser441–Val442) and α2-AP (i.e. Lys12–Leu13 and Ser559–Ile560) are thus in line with this specificity and provide new insights into the putative biological substrates of this staphylococcal protease.

It is of note that besides plasmin, a number of proteases have also been identified that can convert pro-uPA into an active enzyme. Compared with the catalytic efficiency of human plasmin (\(k_{\text{cat}}/K_m = (1.9–7.7) \times 10^5 \text{ s}^{-1} \cdot \text{M}^{-1};\) our results and [39]), however, those reported for other enzymes are lower, ranging from 2.4 \times 10^4 to 4.8 \times 10^4 s^{-1} \cdot M^{-1} for mast cell tryptase and hepsin [39,40] respectively. At 2.6 \times 10^4 s^{-1} \cdot M^{-1}, the specificity constant \(k_{\text{cat}}/K_m\) of aureolysin is thus at the lower end of this range, but equivalent to those reported for acknowledged pro-uPA activators, e.g. for mast cell tryptase and glandular kallikrein (2.4 \times 10^3 and 3.3 \times 10^4 s^{-1} \cdot M^{-1} respectively) [40,41]. Similarly, although aureolysin displays a low affinity for pro-uPA (\(K_m \approx 100 \mu M\) as compared with 2.3 \mu M for plasmin), it is comparable with that determined for tryptase (\(K_m = 34 \mu M\)) [40]. Still, due to this relatively low affinity, aureolysin appears not to be a very efficient pro-uPA activator, particularly at low pro-uPA concentrations such as those in the circulation (1–2 mg/ml). However, pro-uPA concentration increases dramatically in inflammation and infection. Accordingly, up to 0.2 mg/ml of pro-uPA (~4 \mu M, which is the concentration used in our assays) has been measured in lung homogenates of mice infected with Streptococcus pneumoniae, suggesting that locally, pro-uPA concentration may even be higher [42]. Such conditions would favour activation of pro-uPA by aureolysin.

We, therefore, suggest that after colonization by S. aureus and during subsequent inflammation, aureolysin may initiate the auto-amplified proteolytic cascade involving uPA, plasmin and MMP in vivo [2]. This assumption is supported by our observations that (i) aureolysin, either purified or secreted by clinical isolates of S. aureus, indeed activates the human zymogen and (ii) pro-uPA activation by aureolysin occurs in the presence of human plasma. Along with this, other thermolysin-like bacterial proteases, including thermolysin itself, as well as bacilloglysin, both secreted by non-pathogenic bacteria (i.e. Bacillus thermoproteolyticus and Bacillus megaterium respectively), have also been demonstrated to activate pro-uPA [35,43].

Activation of the human fibrinolytic system by bacteria is presumed to support bacterial dissemination and invasiveness (i) through the release of large amounts of bacteria sequestered within fibrin deposits, thus leading to the propagation of infection, and (ii) through hydrolysis of the extracellular matrix and basement membranes, as well as through proteolytic disruption of intercellular contacts, thus provoking tissue destruction [2,6]. Apart from these well-known features, activation of the plasminogen-derived matrilysic system might also damage tissues via induction of cell apoptosis and via amplification of a deleterious immune response, either through the release of matrix-entrapped pro-inflammatory cytokines and chemokines or through the activation of cellular effectors [2,44,45]. Among the array of bacterial factors reported to interact with the plasminogen activation system, the staphylococcal plasminogen activator SAK provides a significant example [5,11]. SAK is a non-proteolytic bacterial protein that forms a stoichiometric complex with plasminogen, resulting in a change of conformation of the latter and facilitating its activation by minute amounts of plasmin, whereas SAK-bound plasmin is protected from interaction with its inhibitor α2-AP [5,11]. SAK, which has attracted major interest because of its putative therapeutic use as a thrombolytic agent [5,11], is thus considered to be a notable bacterial virulence factor. However, in contrast with the

Figure 4  Proteolysis of human plasminogen, PAI-1 and α2-AP by purified aureolysin

(A–C) Plasminogen (Plg), PAI-1 or α2-AP, all at a concentration of 20 \mu g/ml, were exposed to purified aureolysin (Aur) in the range 2.25–40 nM (i.e. E/S molar ratios in the range 1:100 to 1:8), or left untreated as a control, for 1–24 h at 37°C. Alternatively, the proteolytic activity of Aur was blocked with 5 mM EDTA added to the reaction mixture. Proteins were subjected to SDS/PAGE under reducing conditions followed by silver staining. Panels included are representative of independent experiments, and arrows on the left-hand side indicate the location of (A) Plg and Plg-derived molecular species (from the top to the bottom: proteins of ~67.5, 61 and 39.5 kDa), (B) PAI-1 (~43 kDa) or (C) α2-AP and α2-AP-derived molecular species (from the top to the bottom: proteins of ~62 and 53 kDa). (D) Peptide bonds targeted by Aur within Plg were determined by N-terminal microsequencing (FAP, pre-activation peptide, K, kringle domain, C, catalytic domain). Dots indicate the location of amino acid residues constituting the serine protease catalytic triad; the black diamond indicates a N-linked glycosylation site; arrows indicate the position of cleavage sites. (E, F) PAI-1 or α2-AP were exposed, as described above, to 4.5, 22.5 or 45 nM Aur (PAI-1) or to 4 or 40 nM Aur (α2-AP; grey and black bars respectively), or left untreated as a control, for 1 h (PAI-1), for 1–24 h (α2-AP) at 37°C. Aur-processed serpins were then allowed to interact with subsaturating quantities of their respective protease targets, uPA and Pm, for 30 min at room temperature, before measurement of the residual proteolytic activity by using the chromogenic substrates L-1480 and DLVK-902 respectively. Results are expressed as a percentage of inhibitory activity, with 100% corresponding to that of the serpins unexposed to Aur. Histograms represent the means ± S.E.M. for two (E) or three (F) independent experiments.

Although aureolysin’s specificity has not been analysed yet, thermolysin-like enzymes have been demonstrated to preferentially hydrolyse peptide bonds bearing a hydrophobic residue at the P1’ position [38]. The description of limited endopeptidase of fibrinolytic system-related proteins by aureolysin and the identification of the cleavage sites within human macromolecules such as pro-uPA (i.e. Tyr31–Phe35, Lys36–
inhibitors, which have been designed in the context of cancer. As a consequence, the array of therapeutic tools interfering with tumour cells, including uPA overexpression and activation, has inhibitors are worth testing in infectious contexts. Indeed, it is Pseudomonas aeruginosa angiogenic factor angiostatin was recently reported to be an further activated by uPA and by aureolysin-generated uPA, and aureolysin-generated mini-plasminogen retains its capacity to be angiostatin and mini-plasminogen [35,48]. On the one hand, bacterial metalloproteases, aureolysin processes plasminogen into vivo hypothesis, which now demands to be confirmed within an in Listeria monocytogenes and Staphylococcus aureus. We express our gratitude to Sabine Streicher (Department of Clinical REFERENCES


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