Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Comparison of the action of different proteases on virulence properties related to the staphylococcal surface

M. Artini[1]*, R. Papa[1]*, G.L. Scoarughi[1], E. Galano[2], G. Barbato[3], P. Pucci[2] and L. Selan[1]

1 Department of Public Health and Infectious Diseases, Sapienza University, Rome, Italy
2 Department of Organic Chemistry and Biochemistry,Federico II University, Complesso Universitario Monte Sant'Angelo, Naples, Italy
3 Department of Science and Chemical Technologies, University of Rome Tor Vergata, Rome, Italy

Keywords
biofilm, cellular invasion, metalloprotease, serine protease, virulence inhibition.

Correspondence
Laura Selan, Public Health and Infectious Diseases, Sapienza University, Piazzale Aldo Moro 5, 00185 Rome, Italy. E-mail: laura.selan@uniroma1.it

*These authors equally contributed to this work.

2012/1377: received 1 August 2012, revised 19 September 2012 and accepted 4 October 2012

doi:10.1111/jam.12038

Abstract

Aims: The purpose of this study was to evaluate the antimicrobial efficacy of five different proteases belonging to two different families on *Staphylococcus aureus* and *Staphylococcus epidermidis* strains.

Methods and Results: We used three serine proteases and two metalloproteases in single species biofilm formation assays and in human cell invasion processes. Following each protease incubation with bacterial cells, surface protein patterns were analysed by SDS-PAGE and zymography. Some differently expressed proteins were identified by mass spectrometry.

Conclusions: The effect of tested proteases on biofilm formation was not related to the protease category but was strain-dependent and was related to the biofilm formation capacity of each staphylococcal strain. Some proteases showed a nonspecific and indiscriminate effect on surface proteins, while others induced a discrete and reproducible action on protein profiles.

Significance and Impact of the Study: The inhibition of the surface-related virulence factors is a promising avenue to overcome persistent infections caused by bacterial biofilms. To this end, we show here that proteases, in particular the metalloprotease serratiopeptidase, can interfere with adhesion and invasion of eukaryotic cells and biofilm formation in staphylococci and their use could represent a viable treatment for the development of novel combination therapies.

Introduction

Staphylococcus epidermidis and *Staphylococcus aureus*, the most relevant species of their genus, inhabit the skin and mucous membranes of animals and humans. Surface-mediated infectious disease transmission is a major concern in various settings, including schools, hospitals and food-processing facilities. Several surface proteins expressed by these bacteria interact with solid surfaces and are essential in bacterial adhesion and promote, together with exopolysaccharides (EPS), biofilm formation and maturation. In *Staph. aureus*, these proteins also take part in the invasion process of different mammalian cell types. Both species produce various microbial surface proteins recognizing adhesive matrix molecules of eukaryotic cells (MSCRAMMS), as well as other adhesive proteins, which enable bacteria to bind different surfaces moieties. Among them, fibrinogen, collagen and elastin adhesins (*fnbA-B*, *clfA-B*, *cna* and *ebpS*), sialoprotein-binding proteins (*bbp*, *sdrC* and *sdrE*), extracellular matrix-binding proteins (*map*, *eap* and *sasG*), biofilm-associated proteins (*bap*) and adhesins/autolysins (*atl*) were identified (Cucarella *et al.* 2002; Rohde *et al.* 2007). Although biofilm formation in staphylococci typically relies on the production of extracellular polysaccharide adhesin (PIA/PNAG), strains that are able to produce a protein-dependent biofilm were also identified (Götz 2002; O'Gara 2007; Rohde *et al.* 2007; Vergara-Irigaray *et al.* 2009; Christner *et al.* 2010).

Considering the impact of bacterial biofilms on human health, industrial and food-processing activities, the interest in the development of approaches for the prevention

and treatment of staphylococcal adhesion and biofilm formation capabilities has increased. A viable approach should target staphylococcal adhesive properties without affecting bacterial vitality to avoid the rapid appearance of escape mutants. Molecules implicated in active biofilm dispersal include glycosidases, deoxyribonucleases and proteases (Kaplan 2010). Furthermore, the production of extracellular enzymes that degrade adhesive components in the biofilm matrix is a basic mechanism used in the biological competition between phylogenetically different bacteria (Brook 1999; Wang *et al.* 2007, 2010). An inhibitory effect of D-amino acids on *Staph. aureus* biofilm has been recently demonstrated (Hochbaum *et al.* 2011). With the aim of targeting some surface-related virulence features of staphylococci, our first choice was to use proteases. An interesting report by Iwase *et al.* (2010) showed an interesting example of a bacterial exoprotease acting as an antibiofilm and anticolonization agent. In this work, the authors demonstrate that the protease *Esp* secreted by *Staph. epidermidis in vivo* hinders colonization and biofilm formation of *Staph. aureus* cells living in the same ecological niche. Moreover, in the work of Hochbaum *et al.* (2011), it is shown that the action on staphylococcal surface-associated proteins of some D-amino acids resulted in impaired biofilm maturation, while the same amino acids had no effect on EPS. This evidence, together with previous work, gave a significant clue on the importance of surface-associated proteins in the entire process of biofilm development (Hochbaum *et al.* 2011) and not only in initial attachment to the substrate.

In our work, we examined the effect of two families of proteases on *Staph. aureus* and *Staph. epidermidis* strains. We established the following selection criteria: (i) with a relatively small number of proteases, we should explore a large variety of different substrate specificity (i.e. recognition of aromatic, charged, small/large residues, etc.); (ii) the proteases should belong to at least two different enzymatic classes; (iii) they should explore a broad range of molecular weight (to allow for different steric hindrance impact); (iv) they should be monomeric, soluble and function without the need of activating factors and (v) last but still important practically, the proteases should be commercially available. In particular, we used three serine proteases (proteinase K, PK; trypsin, TRY; and chymotrypsin, CHY) and two metalloproteases (serratiopeptidase, SPEP; carboxypeptidase A, CpA) in biofilm formation assays and in human cell invasion processes (invasion only for *Staph. aureus*). Some of these proteases are already utilized in human therapy for different purposes (Martin *et al.* 2002; Hao *et al.* 2006). SPEP is widely used as an anti-inflammatory agent (Kee *et al.* 1989; Mazzone *et al.* 1990), and it has been shown to modulate adhesin expression in some bacterial species and to enhance antibiotic efficacy towards biofilm-forming bacteria (Selan *et al.* 1993; Maheshwari *et al.* 2006; Longhi *et al.* 2008). Our present results show that proteases could represent a viable treatment that could lead to the development of novel combination therapies to prevent staphylococcal colonization and infection.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this work were the following: *Staph. aureus* ATCC 6538P (DSMZ 346), reference strain for antimicrobial testing; *Staph. aureus* ATCC 25923 (DSMZ 1104), clinical isolate; *Staph. aureus* ATCC 12598 (DSMZ 20372), clinical isolate from septic arthritis; *Staph. epidermidis* ATCC 35984 (DSMZ RP62A), reference strain isolated from infected catheter; *Staph. epidermidis* XX-17, clinical isolate; and *Staph. epidermidis* O-47, clinical isolate from septic arthritis kindly provided by Prof. Gotz.

Brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) was used for biofilm formation and planktonic cultures for *Staph. aureus* strains; for *Staph. epidermidis* strains, tryptic soy broth (TSB; Oxoid) was used instead. For all strains, biofilm formation was assessed in static chamber system where planktonic cultures were grown under vigorous agitation both at 37°C.

Cells

HeLa cells were cultured in minimal essential medium with Earle's salts (MEM/EBSS), supplemented with 10% foetal calf serum, 1% glutamine and 1% penicillin–streptomycin in an atmosphere of 95% air and 5% CO_2 at 37°C. All media were from Euroclone. Monolayers were used 48 h after seeding.

Chemicals

SPEP (2540 U mg^{-1}; Takeda Italia Farmaceutici, Rome, Italy), PK (949 U mg^{-1}; Euroclone, Pero, Italy), TRY from bovine pancreas (10 000 U mg^{-1}; Sigma-Aldrich, St Louis, MO) and CHY type I-S from bovine pancreas (58·3 U mg^{-1}; Sigma-Aldrich) were dissolved in PBS and stored at −20°C. Aqueous suspension of CpA from bovine pancreas (1704 U ml^{-1}; Sigma-Aldrich) was stored at 4°C.

Detection of *Staphylococcus aureus* and *Staphylococcus epidermidis* virulence-associated genes

DNA preparation and PCR were performed as follows. Thirty microlitres of each bacterial culture in planktonic

conditions was harvested by centrifugation at 3542 ***g***. The bacterial pellet was washed twice in TE buffer (Tris–HCl 10 mmol l^{-1} pH 8, EDTA 10 mmol l^{-1} pH 8) and resuspended in 100 μl of TE buffer, boiled for 10 min and then centrifuged at 16 627 ***g*** at 4°C to eliminate bacterial debris. Ten microlitres of supernatant containing bacterial DNA partially purified was used for PCR amplifications. Primers and PCR conditions used are summarized in Table 1.

Quantification of biofilm formation in the presence of a protease

Biofilm formation of staphylococcal species was evaluated in the presence of the aforementioned proteases, each at a concentration of 50 U ml^{-1}. Preliminary experiments were carried out to assess the effect of selected proteases on the growth rate of previously described bacterial strains. Cultures were treated with serial dilutions of

Table 1 Primers used for the PCR-based detection of staphylococcal factors involved in the pathogenesis of foreign-body-associated infections

Putative function of encoded protein	Gene	Sequence	References
Staphylococcus epidermidis			
N-acetylglucosaminyltransferase involved in PIA synthesis	*icaR*	5′-TACTGTCCTCAATAATCCCGAA 5′-GGTACGATGGTACTACACTTGATG	Cafiso *et al.* 2004
	icaA	5′-TCTCCCCCTTATTCAATTTTCT 5′-CGATACAATACATCCAAAATACTC	
	icaD	5′-CAGACAGAGGCAATATCCAAC 5′-ACAAACAAACTCATCCATCCG	
	icaB	5′-ATGGCTTAAAGCACACGACGC 5′-TATCGGCATCTGGTGTGACAG	
	icaC	5′-ATCATCGTGACACACTTACTAACG 5′-CTCTCTTAACATCATTCCGACGCC	
Fibronectin adhesin	*embp*	5′-AGCGGTACAAATGTCAAT 5′-AGAAGTGCTCTAGCATCATCC	Rohde *et al.* 2007
Housekeeping gene	*pta*	5′-TTAAAATCGTATTACCTGAAGG 5′-GACCCTTTTGTTGAAAAGCTTAA	Enright *et al.* 2000
Intercellular adhesin	*aap*	5′-AAACGGTGGTATCTTACGTGAA 5′-CAATGTTGCACCATCTAAATCAGCT	Rohde *et al.* 2007
Promotes binding to polystyrol; vitronectin adhesin	*atlE*	5′-CAACTGCTCAACCGAGAACA 5′-TTTGTAGATGTTGTGCCCCA	Rohde *et al.* 2007
Staphylococcus aureus			
N-acetylglucosaminyltransferase involved in PIA synthesis	*icaR*	5′-AGTAGCGAATACACTTCATCTTTGA 5′-GTTGTACCGTCATACCCCTTCTCTG	Cafiso *et al.* 2007
	icaA	5′-CATTGAACAAGAAGCCTGACA 5′-ATATGATTATGTAATGTGCTTGGATG	Cafiso *et al.* 2007
	icaD	5′-ATGGTCAAGCCCAGACAGAG 5′-CGTGTTTTCAACATTTAATGCAA	Arciola *et al.* 2001
	icaB	5′-AGAATCGTGAAGTATAGAAAATT 5′-AGAATCGTGAAGTATAGAAAATT	Kiem *et al.* 2004
	icaC	5′-ACACAGCGTTTCACGATACCG 5′-CCAATAGTCTCCATTTGCTAACGC	Valle *et al.* 2003
Putative adhesin with unknown ligands	*sdrC*	5′-AGCGGTACAAATGTCAAT 5′-GTACTTGAAATAAGCGGTTG	Rohde *et al.* 2007
Fibrinogen adhesin	*clfA*	5′-GTAGGTACGTTAAATCGGTT 5′-CTCATCAGGTTGTTCAGG	Rohde *et al.* 2007
Fibronectin adhesin	*fnbA*	5′-CACAACCAGCAAATATAG 5′-CTGTGTGGTAATCAATGTC	Rohde *et al.* 2007
Housekeeping gene	*gyr*	5′-TTATGGTGCTGGGCAAATACA 5′-CACCATGTAAACCACCAGATA	Wolz *et al.* 2002
Biofilm formation in *Staph. aureus* bovine mastitis isolates	*bap*	5′-CCCTATATCGAAGGTGTAGAATTGCAC 5′-GCTGTTGAAGTTAATACTGTACCTGC	Rohde *et al.* 2007
Adherence to desquamated nasal epithelial cells	*sasG*	5′-CGCGGATTCGCAGCTGAAAACAATATT 5′-CCAAGCTTTAATTCTGTTATTGTTTTTGG	Rohde *et al.* 2007
Autolysin involved in initial adhesion	*atl*	5′-CAGTTAGCAAGATTGCTCAAG 5′-CCGTTACCTGTTTCTAATAGG	Wootton *et al.* 2005

SPEP, PK, TRY, CHY and CpA, respectively (1 : 2 dilutions starting from 200 U ml^{-1}), and bacterial growth was monitored over 24 h. Bacterial growth curves were nearly superimposable both in the presence and in the absence of each protease. Quantification of *in vitro* biofilm production in the presence and in the absence of each protease was based on method previously reported (Artini *et al.* 2011). Briefly, the wells of a sterile 24-well flat-bottomed polystyrene plate were filled with 900 μl of the appropriate medium (BHI for *Staph. aureus* and TSB for *Staph. epidermidis*, respectively). A volume of 100 μl of overnight bacterial cultures was added into each well. The first row contained the untreated bacteria, while each of the remaining rows contained 50 U ml^{-1} of a different single protease per row. The plates were incubated aerobically for 24 h at 37°C. After rinsing with PBS, adhered cells were stained with 0·1% crystal violet, rinsed twice with double-distilled water and thoroughly dried as previously described (Christensen *et al.* 1994). The dye bound to adherent cells was resolubilized with 20% (v/v) glacial acetic acid and 80% (v/v) ethanol per well. The OD of each well was measured at 590 nm. Each data point is composed of three independent samples. Based on the OD per ml obtained from crystal violet coloration, each strain was classified as strong, medium and weak biofilm producer, with appropriate rescaling of the OD range accounting for the different multiwell format utilized in this study (according to the literature, Cafiso *et al.* 2004, 2007).

Adhesion–invasion assays (antibiotic protection assays)

Staphylococcus aureus 6538P from 18-h cultures in BHI broth, grown in the absence of proteases, were further subcultured up to OD_{600} = 0·5 at 37°C in BHI with or without 50 U ml^{-1} SPEP and 50 U ml^{-1} CpA, respectively. HeLa cells were cultured in 24-well plates (BD Falcon, Franklin Lakes, NJ) to obtain semi-confluent monolayers (1×10^5 cells/well) and then were inoculated with 0·05 ml of logarithmic-growing bacterial suspensions at a MOI (multiplicity of infection) of about 10 bacteria per cell. The adhesion–invasion assays were carried out by infecting cells for 1 h at 37°C. Adhesion and invasion were conducted in parallel on twin cellular monolayers. Loosely bound bacteria were removed from the cell monolayers by two washes with PBS. The cells were then lysed with 0·025% Triton X-100 and plated on tryptic soy agar (TSA; Oxoid) to determine viable bacteria (A: cellular adhesion plus invasion). After incubation, to evaluate the infection rates, the monolayers were washed with PBS and 0·5 ml of fresh medium containing 200 μg ml^{-1} of gentamicin was added to each well and maintained for 1 h at 37°C to kill noninternalized bacteria. Cells were then lysed by the addition of 0·025% Triton X-100 and plated on TSA to count viable intracellular bacteria (B: cellular invasion). We further calculated adhesion efficiency by subtraction of B-cfu to A-cfu. Adhesion and invasion efficiency were expressed as percentage of the inoculated bacteria that adhered or invaded HeLa cells, respectively.

Data represent the mean of three independent experiments.

Surface protein extraction and processing

Surface proteins were extracted as follows. Briefly, after centrifugation of 50 ml of each bacterial culture grown in the presence and in the absence of 50 U ml^{-1} of each protease (OD_{600} = 0·6), pellets were washed twice in PBS and then suspended in 500 μl of PBS containing 1% SDS. Samples were incubated at 37°C for 15 min, and after centrifugation, the supernatants were collected and used for further analyses. The protein content was determined by the Bradford procedure (Sambrook and Russell 2001).

SDS-PAGE and zymogram

Equivalent amounts of protein samples were used for each condition (5 μg). SDS-PAGE was carried out by standard methods (Sambrook and Russell 2001) with SDS-polyacrylamide separating gel (10% acrylamide, pH 8·8). Following electrophoresis, proteins were stained with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA). The renaturation of SDS-PAGE was performed as previously reported (Artini *et al.* 2011). Briefly, SDS-PAGE was prepared adding 0·2% (w/v) lyophilized *Micrococcus luteus* cells provided by Sigma, to detect the lytic activities. After electrophoresis, the gels were soaked and then transferred into the renaturing buffer (50 mmol l^{-1} Tris–HCl, pH 8·0, containing 1% Triton X-100) and shaken for 2 h at 37°C. The renatured autolysins appeared as clear translucent bands on opaque background. For each experiment, two gels were simultaneously prepared and electrophoresed. No difference was noted in the migration of the standards because of the presence of *M. luteus* cells in the gel.

In situ digestion and protein identification

Protein identification following *in situ* digestion was performed as previously reported (Artini *et al.* 2011). Briefly, protein bands were excised from the gel and washed; protein samples were reduced, and free cysteines were alkylated by incubation with 10 mmol l^{-1} DTT and 55 mmol l^{-1} iodoacetamide, respectively. After wash, gel particles were digested with trypsin. Peptides were

extracted and then analysed by LC-MS/MS using a CHIP-QTOF 6520 equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA, USA). After loading, each peptide mixture was first concentrated, washed and then fractionated on a C18 reverse-phase capillary column (75 μm × 43 mm in the Agilent Technologies chip). Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 2000 *m*/*z*) followed by MS/MS of the three most abundant ions using in-house MASCOT software (Matrix Science, Boston, MA, USA).

Results

Identification of genes involved in adhesion and correlation with biofilm production

In staphylococci, bacterial adhesion and biofilm formation depend on a complex interplay of adhesins comprising fibrinogen-binding proteins (FnBP-A/B, Embp and ClfA), sialoprotein-binding proteins (SdrC), extracellular matrix-binding proteins (SasG), biofilm-associated protein (Aap and Bap), proteins involved in PIA synthesis (IcaADBC), autolysins (Alt and AtlE), etc. (Rohde *et al.* 2007). The six staphylococcal strains here considered (see Material and Methods section) were investigated by PCR to assess the presence of genes coding for various proteins involved in adhesion and biofilm formation. Results are summarized in Table 2. Two of three *Staph. epidermidis* strains were positive for genes involved in PIA synthesis (XX-17 strain is negative for the presence of entire *ica* locus). Furthermore, all strains were positive for *atlE*, *aap* and *embp* genes coding for adhesins. Determination of biofilm formation showed a strong production for the O-47 strain (2·08 ± 0·11), medium/strong production for the reference strain ATCC35984 (0·74 ± 0·07) and a medium/weak biofilm formation for the XX-17 strain (0·35 ± 0·02).

From a genetic point of view, the three *Staph. aureus* strains are identical for the presence of *ica* operon and adhesion genes. The biofilm-forming ability of *Staph. aureus* strains was tested by quantitative assay. They showed different capabilities to form biofilm that can be schematized as reported: ATCC 6538P is a strong biofilm producer (1·65 ± 0·15), ATCC 25923 is a medium/strong biofilm producer (0·79 ± 0·21), and ATCC 12598 is a medium/weak biofilm producer (0·22 ± 0·03).

Effect of two different classes of protease on staphylococcal biofilm formation

The effect of each protease was related to the biofilm formation capacity of the staphylococcal strains here considered. Results are summarized in Fig. 1 and Table 3.

The inhibition of biofilm formation ranges from almost total (approx. 5% of residual biofilm formation) to just slight inhibition (approx. 90%). Taking into account wide fluctuation in biofilm formation among three replicates of the same experimental set, in the range of 90–110% biofilm formation, the process was considered essentially not affected, being this value the same as the control. When the value was >110%, the action of the protease was considered as favouring biofilm formation. The statistical significance of effect obtained from each protease treatment was calculated with unpaired *t*-Student test.

TRY and CpA hampered biofilm formation, in some cases slightly, of three bacterial strains, PK of 4, CHY of 5 and only SPEP showed inhibiting activity of all the tested strains (see Fig. 1 and Table 3).

The inhibition of biofilm formation among strains was compared. Inhibition in *Staph. aureus* strains was slightly more pronounced than that in *Staph. epidermidis*, as shown in Fig. 1, with a stronger inhibition exerted on the best biofilm former *Staph. aureus* 6538P. In *Staph. aureus*, CHY and SPEP exhibited the stronger inhibition capability (36 and 59% biofilm formation on average, respectively), while PK had a weak inhibition effect (69% biofilm formation), TRY did not show any effect (107% biofilm formation on average), and CpA displayed an average favouring effect (approx. 140% biofilm formation). In *Staph. epidermidis*, SPEP, PK and CHY showed an almost equivalent inhibitory action with values ranging 70–80% biofilm formation, TRY had no effect, while CpA again exhibited a favouring effect (approx. 240%).

SPEP action seemed to be proportional to the ability to form biofilm of each tested strain. The esometalloprotease CpA showed a good effect on stronger biofilm producers (*Staph. aureus* ATCC 6538P, ATCC 25923 and *Staph. epidermidis* O-47) but, conversely, seemed to favour biofilm formation in the case of medium and weak biofilm producers (*Staph. aureus* ATCC 1298, *Staph. epidermidis* ATCC 35984 and XX-17). The three serine proteases showed different behaviour on each bacterial strain, inhibiting and/or facilitating biofilm formation (Fig. 1).

Analysis of surface protein pattern following the treatment with proteases

Cell surface protein samples from treated and untreated cultures of all the strains were simultaneously analysed by SDS-PAGE and zymogram assays. *Staph. aureus* ATCC 6538P was chosen as representative strain because it is a strong biofilm producer and it showed a better response to treatment with proteases. Fig. 2 shows the SDS

Table 2 Genotype characterization of staphylococcal strains

	Staphylococcus aureus			*Staphylococcus epidermidis*		
Putative function of encoded protein	ATCC 6538P (DSMZ 346)	ATCC 25923 (DSMZ 1104)	ATCC 12598 (DSMZ 20372)	O-47	ATCC 35984 (DSMZ RP62A)	XX-17
Biofilm production	Strong	Medium	Weak	Strong	Medium	Weak
N-acetylglucosaminyltransferase involved in PIA synthesis (*ica locus*)	+	+	+	+	+	−
Autolysin gene involved in initial adhesion (*atl*)	+	+	+	+	+	+
Fibronectin adhesin (*fnbA*)	+	+	+			
Putative adhesin with unknown ligands (*sdrC*)	+	+	+			
Fibrinogen adhesin (*clfA*)	+	+	+			
Intercellular adhesin (*aap*)				+	+	+
Fibronectin adhesin (*embp*)				+	+	+
Involved in biofilm formation in *Staph. aureus* bovine mastitis isolates (*bap*)	−	−	−			
Adherence to desquamated nasal epithelial cells (*sasG*)	−	−	−			

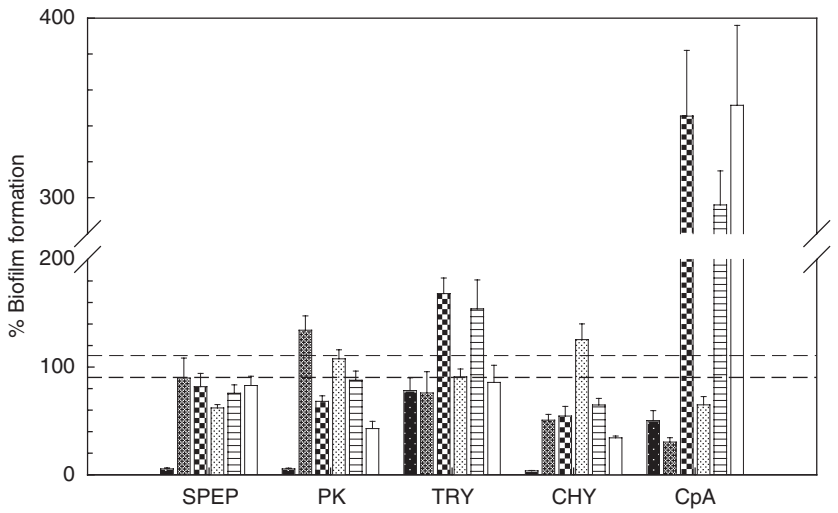

Figure 1 Effect of protease treatment on biofilm formation for each staphylococcal strain. Data are reported as percentage of residual biofilm formation in the presence of each protease in comparison with the untreated one (100%). Biofilm formation was considered unaffected in the range 90–110%. Each data point represents the mean ± SD of three independent experiments. SPEP, serratiopeptidase; PK, proteinase K; TRY, trypsin; CHY, chymotrypsin; CpA, carboxypeptidase A; *Staphylococcus aureus* ATCC 6538P; *Staph. aureus* ATCC 25923; *Staph. aureus* ATCC 12598; *Staph. epidermidis* ATCC 35984; *Staph. epidermidis* XX-17; *Staph. epidermidis* O-47. (▩) 6538P; (▩) 25923; (▩) 12598; (▩) O-47; (▤) 35984 and (□) XX-17.

electrophoretic profiles of the protein mixtures obtained from surface protein extracts of *Staph. aureus* ATCC 6538P following colloidal Coomassie blue staining (left panel) and after the autolytic pattern analysis (right panel).

Several discrete protein bands corresponding to the surface proteins extracted from untreated *Staph. aureus* 6538P cells were present in the control lane 1. This profile was compared with the protein patterns obtained after protease treatment (lanes 2–6).

Metalloprotease treatments (lanes 2 and 6) induced a light modification of surface protein patterns and CpA seemed to remain anchored to the bacterial surface producing the most intense band at about 28 kDa in lane 6. A number of protein bands visible in the untreated *Staph. aureus* 6538P protein profiles either disappeared or

Table 3 Effect of protease treatment on staphylococcal biofilm formation

	NT	SPEP	PK	TRY	CHY	CpA
Staphylococcus aureus						
ATCC 6538P	1·647 ± 0·150	0·086 ± 0·015	0·090 ± 0·012	1·287 ± 0·196	0·059 ± 0·006	0·825 ± 0·152
ATCC 25923	0·793 ± 0·206	0·711 ± 0·146	1·061 ± 0·105	0·604 ± 0·157	0·397 ± 0·043	0·240 ± 0·032
ATCC 12598	0·227 ± 0·032	0·179 ± 0·027	0·146 ± 0·011	0·372 ± 0·032	0·122 ± 0·020	0·756 ± 0·080
Staphylococcus epidermidis						
O-47	2·088 ± 0·108	1·295 ± 0·067	2·241 ± 0·174	1·885 ± 0·151	2·611 ± 0·304	1·352 ± 0·161
ATCC 35984	0·744 ± 0·076	0·563 ± 0·058	0·652 ± 0·063	1·140 ± 0·199	0·476 ± 0·044	2·189 ± 0·141
XX-17	0·354 ± 0·022	0·290 ± 0·030	0·148 ± 0·023	0·300 ± 0·056	0·121 ± 0·006	1·277 ± 0·163
P-value		<0·05	<0·05	<0·001	<0·001	<0·05

NT, nontreated samples; SPEP, serratiopeptidase; PK, proteinase K; TRY, trypsin; CHY, chymotrypsin; CpA, carboxypeptidase A.
Based on OD absorbance at 590 nm. Data represent the mean ± SD of three independent experiments.
Statistical significance is calculated with unpaired *t*-Student test.

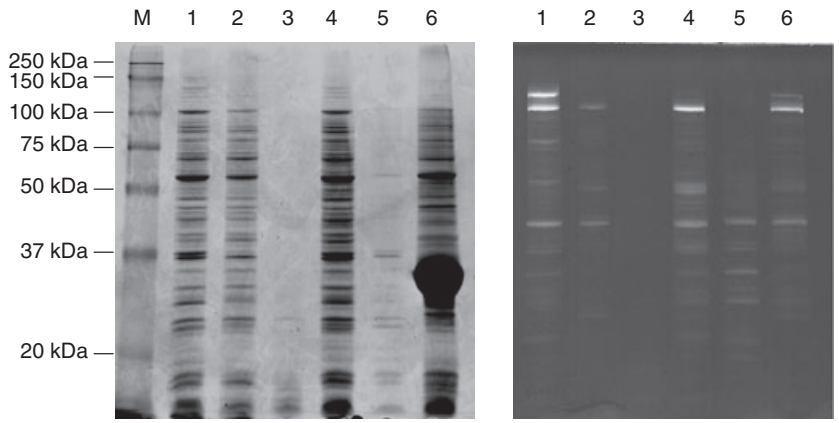

Figure 2 SDS-PAGE and zymogram analyses of *Staphylococcus aureus* 6538P surface proteins. Crude cell envelope SDS extracts from treated and untreated (control) cells were analysed by SDS-PAGE (left panel) and zymogram assay (right panel). The apparent molecular masses of standard indicated are in kDa. Autolysins formed translucent areas in the zymogram. *M*, Molecular weight marker (Bio-Rad); 1, untreated sample; 2, serratiopeptidase-treated sample; 3, proteinase K-treated sample; 4, trypsin-treated sample; 5, chymotrypsin-treated sample; 6, carboxypeptidase A-treated sample.

were drastically reduced in intensity after incubation with the proteases. The protein bands present in the untreated *Staph. aureus* protein profile and disappearing upon SPEP and CpA treatments were selected for further analyses (see below).

PK and CHY treatments (lanes 3 and 5) induced a nearly complete digestion of most of the surface proteins, suggesting a nonspecific and indiscriminate effect of these proteases. These data could confirm the strong effect of the two serine proteases as biofilm inhibitors. TRY did not exhibit any major influence on the surface protein pattern of *Staph. aureus* ATCC 6538P, thus in agreement with its neutral effect on biofilm formation (lane 4).

The autolytic profiles of treated and untreated surface proteins extracted from *Staph. aureus* 6538P were in accordance with the results observed from SDS-PAGE analysis. Treatment with SPEP and CpA led to the disappearance of a discrete number of autolysin bands. On the contrary, treatment with PK led to a complete digestion of surface autolysins while, following treatment with CHY, a shift from high molecular weight to low molecular weight of specific autolytic bands was observed. This latter was in accordance with the results obtained from SDS-PAGE analysis.

Effect of proteases on *Staphylococcus aureus* adhesion to and invasion of HeLa cells

The adhesion efficiency of untreated *Staph. aureus* 6538P was about 2·75% ± 0·45, while $0{\cdot}7 \times 10^{-2}\% \pm 0{\cdot}1 \times 10^{-2}$ of these bacteria invaded HeLa cell line.

The ability of each protease to interfere with *Staph. aureus* capacity to adhere and invade human cells was tested by antibiotic protection assay on HeLa cell

line. Preliminary experiments were addressed to assess the cytotoxicity of each protease on HeLa cells using the same protease concentrations adopted in the previous assays. Cell morphology, viability and proliferation remained unaffected by SPEP and CpA treatments. In contrast, incubation with PK, TRY and CHY strongly affected morphology, viability and proliferation of HeLa cells, which detached from the solid support. According to these data, the effect of the proteases on *Staph. aureus* adhesion to and invasion of eukaryotic cells was analysed only in the presence of SPEP and CpA. *Staph. aureus* ATCC 6538P was chosen as reference strain because in previous work, it showed proper invasion capability (Artini *et al.* 2011).

Figure 3 shows the adhesion and invasion efficiency of SPEP-treated and CpA-treated bacteria compared with untreated control cells. Our results showed that the adhesion efficiency of *Staph. aureus* 6538P was only slightly affected by enzymatic incubation with SPEP (reduction approx. 20%), while it was strongly influenced by treatment with CpA (reduction > 85%) (Fig. 3a). On the contrary, the invasion efficiency was drastically reduced (about 200-fold) following SPEP treatment, while it was reduced by incubation with CpA of approx. 52%.

Identification of surface proteins affected by SPEP and CpA treatment

The duration of the electrophoretic run was extended to achieve a better separation of high molecular weight protein bands. The protein bands occurring in the untreated *Staph. aureus* SDS-PAGE protein profile and disappearing upon SPEP and CpA treatments (numbered from one to eight in Fig. 4) were excised from the gel and submitted to identification by mass spectrometric methodologies. The corresponding gel slices from SPEP-treated sample and CpA-treated sample lanes were also excised and submitted to the identification procedure as control. Proteins identified by mass spectrometric analyses are listed in Table 4.

Discussion

Recently, experimental evidences suggest that in *Staph. epidermidis*, the proteinaceous intercellular adhesin Embp is sufficient and necessary for biofilm formation (Christner *et al.* 2010). Embp is a 1-MDa giant surface protein that mediates attachment to host extracellular matrix, biofilm accumulation and escape from phagocytosis and appears to be well suited for promoting implant-associated infections. Another example of proteinaceous adhesin is the accumulation-associated protein Aap, identified as a polysaccharide-independent intercellular adhesin mediating *Staph. epidermidis* biofilm formation (Rohde *et al.* 2007).

In the light of these considerations, we sought for a proof of concept about the use of proteases for the inhibition of staphylococcal virulence related to the bacterial surface. To this end, we examined the effect of two classes of proteases on different *Staph. aureus* and *Staph. epidermidis* strains. It has been shown by us and others that SPEP is effective in preventing experimental infections caused by biofilm-forming bacteria and enhances antibiotic efficacy (Selan *et al.* 1993; Maheshwari *et al.* 2006; Longhi *et al.* 2008). None of the tested proteases showed an effect on the planktonic growth rate of the staphylococcal strains analysed. This result was in accordance with previous work (Selan *et al.* 1993).

We compared the biofilm formation with the genetic background of the strains here analysed. In *Staph. aureus*, *ica* operon alone is not sufficient to account for high

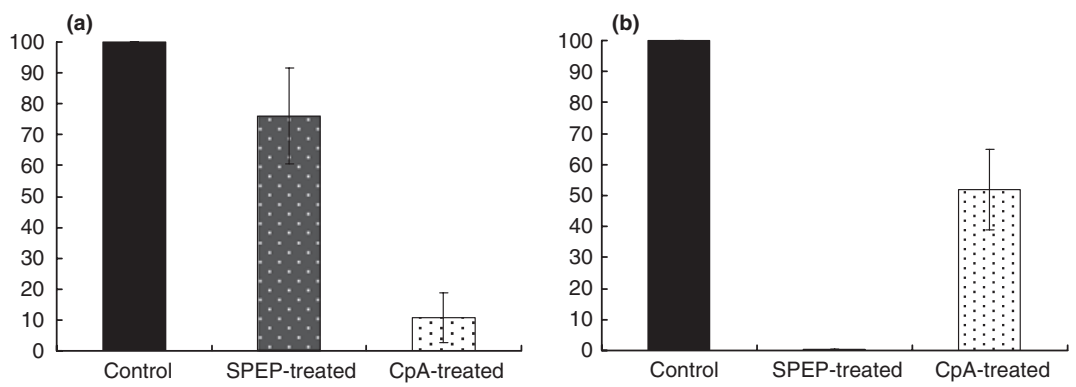

Figure 3 Adhesion and invasion capabilities of serratiopeptidase- and carboxypeptidase A-treated *Staphylococcus aureus* 6538P bacteria in comparison with untreated bacteria. (a) shows the percentage of adhered bacteria compared with the control (untreated sample). (b) reports the percentage of internalized bacteria in HeLa cells compared with the control (untreated sample).

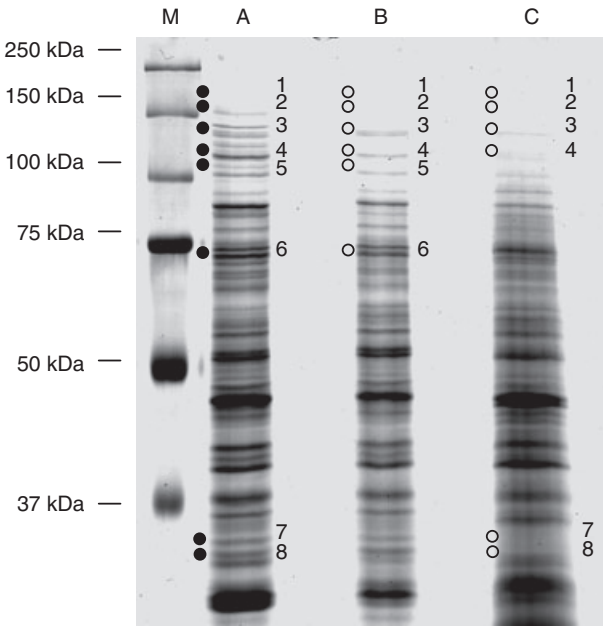

Figure 4 Crude cell envelope SDS extracts from (a) untreated, (b) serratiopeptidase- and (c) carboxypeptidase A-treated *Staphylococcus aureus* 6538P cells analysed by SDS-PAGE. Protein bands indicated with black dots and numbered from one to eight, which were present in the untreated samples and completely disappeared following enzymatic treatment, were identified by mass spectrometric analysis. Gel slices from treated sample lanes corresponding to the selected one to eight bands (empty dots) were also submitted to the identification procedure as control.

biofilm production, whereas in *Staph. epidermidis*, its absence seems to correspond to a lower biofilm production (Table 2). There is no evident correlation between the genetic background of the staphylococcal strains here tested and protease effectiveness. Considering a single strain, the best antibiofilm performance was obtained using CHY. All tested serine proteases showed different behaviour on each bacterial strain. In *Staph. aureus*, PK and CHY displayed a strong *in vitro* effect as biofilm inhibitors. However, their action induced a nearly complete digestion of most of surface proteins, suggesting a nonspecific and indiscriminate effect of these proteases. On the contrary, CpA treatment enhanced biofilm formation in three of six tested strains (one *Staph. aureus* strain and two *Staph. epidermidis* strains). This closely recalls the effect found for GelE, a metalloprotease produced by *Enterococcus faecalis*, which belongs to the thermolysin M4 family (Hancock and Perego 2004; Thomas and Hancock 2009). CpA enzymatic function shows mechanistic similarities with thermolysins (Adekoya and Sylte 2009). Interestingly, GelE is required for the formation of *Ent. faecalis* biofilm and increases biofilm accumulation by a sophisticated fratricide mechanism (Waters *et al.* 2003). Cell death and lysis have been ascribed to play important biological roles in bacterial developmental processes, including biofilm development, by means of a rapid release of adhesive moieties, among them proteins anchored to the cytoplasmic side of the membrane. This mechanism was found to regulate the biofilm phenotype

Table 4 Proteins identified by mass spectrometry analysis in untreated, SPEP- and CpA-treated surface protein samples from *Staphylococcus aureus* 6538P

Band	MW		Untreated protein	SPEP-treated protein	CpA-treated protein	Accession number
1	133 504	DNA-directed RNA polymerase subunit beta	+	–	–	Q932F8
2	137 339	Autolysin, N-acetylmuramyl-L-alanine amidase	+	–	–	Q931U5
3	117 554	Carbamoyl phosphate synthase large subunit	+	–	–	P63739
3	99 213	DNA gyrase, A subunit	–	–	+	Q932M0
4	89 113	Phenylalanyl-tRNA synthetase beta chain	+	–	na	P67040
4	96 114	SecA1 translocase	+	–	+	Q99VM2
5	63 876	Prolyl-tRNA synthetase	+	–	na	Q99UK9
5	66 728	Aspartyl-tRNA synthetase	+	–	na	P67014
5	39 890	Cell division protein ftsA	+	–	na	P63764
6	38 850	Uncharacterized protein SAV1921	+	na	–	Q99SW6
6	33 465	Aspartate carbamoyltransferase catalytic subunit	+	na	–	P65617
7	36 266	Branched chain alpha ketoacid DH E1	+	na		Q99tx7
8	33 021	Fructose-1,6-bisphosphate aldolase	–	na	+	P67472
8	34 047	Ferric hydroxamate receptor 1	–	na	+	Q99RY8
8	52 899	Staphylocoagulase	–	na	+	Q99WZ3

SPEP, serratiopeptidase; CpA, carboxypeptidase A; +, protein present in the sample analysed; –, protein not present in the sample analysed; na, the sample was not analysed.

also in *Pseudomonas aeruginosa* and *Staph. aureus* (Thomas and Hancock 2009).

SPEP treatment resulted in an effective and broad-spectrum reduction in biofilm formation. Moreover, its action appears to be more selective, sequence specific (Miyata *et al.* 1970a,b) and proportional to biofilm production (this study).

The use of PK, TRY and CHY in antibiotic protection assay strongly affected morphology, viability and proliferation of HeLa cells, while SPEP and CpA did not. For these reasons, only SPEP and CpA were considered for further investigations. SPEP treatment strongly impaired *Staph. aureus* invasion efficiency of HeLa cells. This is similar to what found for *Listeria monocytogenes* (Longhi *et al.* 2008), thus confirming the broad spectrum of this protease also against other virulence properties different from biofilm formation. CpA displayed a strong inhibitory effect only on the adhesion efficiency, and this could be related to the CpA ability to bind bacterial surface as shown by SDS-PAGE analysis. The presence of CpA may exert a steric effect masking bacterial proteins involved in the adhesion to the host cells. The remaining CpA-treated bacteria that retain the capability to adhere to host cells might still be able to invade them.

Comparative proteomic investigations on SPEP-treated, CpA-treated and untreated *Staph. aureus* 6538P cells revealed a number of proteins affected by the enzymatic treatment (Table 3). SPEP and CpA treatment showed a specific and reproducible action on bacterial cells, leading to the disappearance of discrete protein bands in the SDS-extracted protein pattern. Among the negatively regulated proteins, some surface proteins like the adhesin/autolysin N-acetylmuramyl-L-alanine amidase, SecA1 translocase that has a role in the transfer of proteins into and across the cell membrane and the cell division protein FtsA were identified (Table 3). The biofilm production, the adhesion and the invasion capabilities (mainly in *Staph. aureus*) are essential virulence factors of staphylococci that directly or indirectly are related to proteins located on the bacterial surface. Extensive functional roles are played by adhesins that show high affinity for eukaryotic epithelial structures and are also involved in numerous cellular processes including anchoring to a substrate and biofilm formation (Cucarella *et al.* 2001; Foster 2005; Clarke and Foster 2006). Surface proteins are then critically important in determining the success of bacterial strains in their competition for survival (Navarre and Schneewind 1999; Roche *et al.* 2003; Pinheiro and Ellar 2006; Tjalsma *et al.* 2008).

The other proteins affected by the protease treatment are strictly cytoplasmic proteins, like the nucleic acid-binding proteins, DNA-directed RNA polymerase subunit beta and the DNA gyrase A subunit, or the t-RNA synthetases (phenylalanil, aspartyl and prolyl), or the aspartate carbamoyl transferase and carbamoyl phosphate synthetase subunits. A number of hypotheses can be drawn from these results. For example, the protease action could induce bacterial lysis or could imply its internalization within the bacteria. However, these two hypotheses can be ruled out because we did not evidence an effect on the bacterial growth rate.

A third and to our opinion most likely hypothesis assumes that protease action on cytoplasmic proteins can occur only on a subpopulation of cells deputed to suicidal behaviour. The altruistic suicide mechanism was found to be responsible for the lysis of a bacterial subfraction by *Staph. aureus* biofilm (Rice *et al.* 2007). SPEP and CpA are able to act both on surface adhesins and on the lysed cellular debris derived from the suicidal subpopulation. While the action of CpA, analogously to that of GelE, results in an increase in biofilm accumulation, the action of SPEP is impairing both surface adhesins/autolysins and the adhesive moieties of the altruistic suicidal cells. It is important to underline that SPEP neither influences bacterial viability when used at the concentrations adopted in this work and at higher concentrations nor displays a cytotoxic effect on eukaryotic cell lines (this study, Longhi *et al.* 2008).

The present study was intended at obtaining a broader knowledge regarding the possible use of proteases as anti-adhesive molecules whose use could be proposed in combination therapy with antibiotics. In this respect, among the tested proteases, SPEP seems the most promising molecule to be developed as a novel antivirulence tool. Its action selectively affects a discrete number of proteins clearly involved in fundamental mechanisms associated with bacterial virulence, such as adhesion, invasion and biofilm formation and would thus hinder staphylococcal virulence properties.

Acknowledgements

The Authors thank Dr A. Cellini for his precious technical support.

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