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A new anti-infective strategy to reduce the spreading of antibiotic resistance by the action on adhesion-mediated virulence factors in *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a flexible microbial pathogen frequently isolated from community-acquired and nosocomial infections. *S. aureus* expresses a wide array of secreted and cell surface-associated virulence factors, including proteins that promote adhesion to damaged tissue and to the surface of host cells, and that bind proteins in blood to help evade immune responses. Furthermore, surface proteins have a fundamental role in virulence related properties of *S. aureus*, including biofilm formation. The present study evaluates the anti-infective capabilities of a secreted protein of *Serratia marcescens* (serratiopeptidase, SPEP), in impairing some staphylococcal virulence-related properties, such as attachment to inert surfaces and adhesion/invasion on eukaryotic cells. SPEP seems to exert its action by modulating specific proteins. It is not assessed if this action is due to the proteolytic activity of SPEP or to a specific mechanism which triggers an out/inside signal. Proteomic studies performed on surface proteins extracted from SPEP treated *S. aureus* cultures revealed that a number of proteins are affected by the treatment. Among these we found the adhesin/autolysin Atl, SdrD, Sbi, EF-Tu and EF-G. EF-Tu and EF-G are known to perform a variety of function, depending on their cytoplasmic or surface localization. All these factors can facilitate bacterial colonization, persistence and invasion of host tissues. Our results suggest that SPEP could be developed as a potential "anti-infective agent" capable to hinder the entry of *S. aureus* into human tissues, and also impairs the ability of this pathogen to adhere to prostheses, catheters and medical devices.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a flexible microbial pathogen frequently isolated from community-acquired and nosocomial infections [1,2]. This microorganism can also be found as a part of the normal human resident flora and up to two-thirds of the healthy population are permanently or transiently colonized by *S. aureus* [3]. The rapid emergence of hospital associated, antibiotic resistant *S. aureus* strains is a major epidemiological problem worldwide [4,5]. Moreover, the increased use of medical devices is associated with a significant risk of intravascular and systemic infections by staphylococci, which frequently causes persistent infections on catheters, shunts, vascular and orthopedic prostheses, and other implanted devices [6–9]. The ability of *S. aureus* to adhere on both eukaryotic cells and abiotic surfaces via cell wall proteins and to form biofilm are important virulence factors in chronic infections associated with implanted biomaterials, which are particularly difficult to eradicate [10–13].

Hence, not surprisingly, the interest in the development of alternative anti-infective approaches for the prevention and treatment of staphylococcal infections has increased in recent years [14–17]. A successful strategy should not affect processes essential for bacterial survival in order to avoid the rapid appearance of escape mutants. An innovative approach should target *S. aureus* major virulence factors without affecting bacterial viability.

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Biofilm formation requires two main steps: adherence of bacterial cells to a surface and accumulation of multilayered cell clusters. Although the best-understood biofilm mechanism in staphylococci is the production of an extracellular polysaccharide adhesin (PIA/PNAG), analysis of the factors that assemble cells into a biofilm has revealed the occurrence of strains that produce either PIA/PNAG − or a protein-dependent biofilm [6,18].

During the primary attachment the bacteria make contact with the surface, mediated in *S. aureus* and *Staphylococcus epidermidis* by specific adhesins. Thus during this phase proteins play a critical pivotal role. In the second accumulative phase, biofilm formation, most staphylococci have no direct contact with the surface but remain in the biofilm via expression of intercellular adhesive mechanisms [19]. Recently, it has been shown that, besides their best-known role in the eukaryotic invasion process, fibronectin binding proteins (FnBPs) play a relevant role in the biofilm-associated foreign-body infections. Interestingly, FnBPs promote biofilm formation at the level of intercellular accumulation and not primary attachment [18].

With the aim of targeting some surface-related virulence features of staphylococci our first choice was to use a protease. In literature is reported the in vivo effect of the protease Esp secreted by *S. epidermidis* acting as an anti-biofilm and anti-colonization agent against *S. aureus* cells living in the same ecological niche [20]. In this work we focused our attention on serratiopeptidase (SPEP), an extracellular metalloprotease produced by the Gram-negative opportunistic pathogen *Serratia marcescens* [21,22]. SPEP is commonly used as an anti-inflammatory agent [23,24] and it has been shown to modulate adhesin expression in some bacterial species and to enhance antibiotic efficacy towards biofilm-forming bacteria [25–28].

Previous work demonstrated that SPEP is effective in preventing experimental infections caused by biofilm-forming bacteria and enhances antibiotic efficacy [27,28]. The effect of SPEP treatment on virulence properties of different *S. aureus* strains, such as attachment to inert surfaces and adhesion/invasion of eukaryotic cells, was investigated. Although SPEP did not affect bacterial viability, it is able to impair attachment to abiotic surface and invasiveness by *S. aureus* to human cells and affects both biofilm formation and biofilm dispersion. Furthermore, we identified bacterial proteins affected by SPEP treatment, by using a proteomic approach. Some of identified proteins are known to be multifunctional factors involved in metabolic pathways, in adhesion to extracellular matrix and invasion of host cells, such SdrD, EFTu, EF-G, Atl, SsA2 and Sbi.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this work were: *S. aureus* ATCC 6538P (DSMZ 346), reference strain for antimicrobial testing; *S. aureus* ATCC 25923 (DSMZ 1104), clinical isolate; *S. aureus* ATCC 12598 (DSMZ 20372), clinical isolate from septic arthritis; *S. aureus* ATCC BAA1556 (FPR3757 strain) is a USA 300 strain [29].

Brain heart infusion broth (BHI, Oxoid, UK) medium was used for biofilm formation in static chamber system at 37 °C. Tryptic soy broth (TSB, Oxoid, UK) was used for biofilm formation in BioFlux system. Planktonic culture was grown in BHI at 37 °C under vigorous agitation (180 rpm).

2.2. Cells

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

2.3. Chemicals

Serratiopeptidase (SPEP, 2540 U mg^{-1}), obtained from Takeda Italia Farmaceutici (Rome, Italy), was dissolved in phosphate-buffered saline pH 7.2 (PBS) at a stock concentration of 20,000 U mL^{-1} and stored at −20 °C. Proteinase k (PK, 949 U mg^{-1}, Euroclone) was dissolved in PBS and stored at −20 °C. TPCK-treated trypsin, dithiothreitol (DTT), iodoacetamide and alfa-cyano-4-hydroxycinnamic acid were purchased from Sigma. Ammonium bicarbonate was from Fluka. Trifluoroacetic acid (TFA)-HPLC grade was from Carlo Erba. All other reagents and solvents were of the highest purity available from Baker.

2.4. Detection of S. aureus virulence associated genes

DNA preparation and PCR were performed as follows. 30 μL of bacterial cultures grown overnight in planktonic condition were harvested by centrifugation at 6000 rpm for 10 min. The bacterial pellet was washed twice in TE buffer (Tris—HCl 10 mM pH 8, EDTA 10 mM pH 8) and centrifuged at 6000 rpm at 4 °C for 10 min, to eliminate growth medium. Bacterial pellet was resuspended in 100 μL of TE buffer, boiled for 10 min at 100 °C and then centrifuged at 13,000 rpm at 4 °C for 10 min to eliminate bacterial debris. 10 μL of supernatant containing bacterial DNA partially purified was used for PCR amplifications. Primers and PCR conditions are summarized in Table 1.

2.5. MIC assays

MICs by SPEP were determined in 96-well plates measuring the optical density at the wavelength of 600 nm (OD600). BHI broth was added to all wells and SPEP (1000 U mL^{-1}) was added to the first well and serially diluted (1:2 dilutions). Logarithmic-phase cultures of *S. aureus* were added to each well to achieve 10^6 CFU/well. The microtitre plates were incubated at 37 °C and OD600 was recorded after 1 and 24 h. The MICs were defined as the lowest concentrations of SPEP that completely inhibited growth.

2.6. Quantification of biofilm formation

2.6.1. Static biofilm assay

Quantification of *in vitro* biofilm production was based on the method described by Christensen [30]. The wells of a sterile 96-well flat-bottomed polystyrene plate (Falcon) were filled with 90 μL of the appropriate medium containing or not containing the inhibitors. 10 μL of overnight bacterial cultures grown in BHI was added into each well. The plates were incubated aerobically with or without the enzyme for 24 h at 37 °C. Growth was monitored by measuring the OD600, and after 24 h incubation the ability of the *S. aureus* strain to adhere to the polystyrene plates was tested. The content of the plates was then poured off and the wells washed with sterile distilled water. The plates were then stained with crystal violet for 5 min. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the 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. A dose-dependent effect on biofilm formation was performed adding SPEP 800 U mL^{-1} to the first well and serially diluted (1:2 dilutions). The concentration adopted for further experiments was 200 U mL^{-1}.

2.6.2. Dynamic biofilm assay

To continuously monitor biofilm development in dynamic condition, we utilized a BioFlux 2000 microfluidics system (Fluxion

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

Putative function of encoded protein	Gene	Sequence	Ref.
N-Acetylglucosaminyltransferase involved in PIA synthesis	*icaR*	5′-AGTAGCGAATACACTTCATCTTTGA 5′-GTTGTACCGTCATACCCCTTCTCTG	[29]
	icaA	5′-CATTGAACAAGAAGCCTGACA 5′-ATATGATTATGTAATGTGCTTGGATG	[29]
	icaD	5′-ATGGTCAAGCCCAGACAGAG 5′-CGTGTTTTCAACATTTAATGCAA	[59]
	icaB	5′-AGAATCGTGAAGTATAGAAAATT 5′-AGAATCGTGAAGTATAGAAAATT	[60]
	icaC	5′-ACACAGCGTTTCACGATACCG 5′-CCAATAGTCTCCATTTGCTAACGC	[61]
Putative adhesin with unknown ligands	*sdrC*	5′-AGCGGTACAAATGTCAAT 5′-GTACTTGAAATAAGCGGTTG	[19]
Fibrinogen adhesin	*clfA*	5′-GTAGGTACGTTAAATCGGTT 5′-CTCATCAGGTTGTTCAGG	[19]
Fibronectin adhesin	*fnbA*	5′-CACAACCAGCAAATATAG 5′-CTGTGTGGTAATCAATGTC	[19]
Housekeeping gene	*gyr*	5′-TTATGGTGCTGGGCAAATACA 5′-CACCATGTAAACCACCAGATA	[62]
Biofilm formation in *S. aureus* bovine mastitis isolates	*bap*	5′-CCCTATATCGAAGGTGTAGAATTGCAC 5′-GCTGTTGAAGTTAATACTGTACCTGC	[19]
Adherence to desquamated nasal epithelial cells	*sasG*	5′-CGCGGATTCGCAGCTGAAAACAATATT 5′-CCAAGCTTTAATTCTGTTATTGTTTTTGG	[19]
Autolysin involved in initial adhesion	*atl*	5′-CAGTTAGCAAGATTGCTCAAG 5′-CCGTTACCTGTTTCTAATAGG	[63]

Biosciences Inc., San Francisco, CA), which allows the acquisition of microscopic images over time. To grow biofilm in the BioFlux system, the channels were first primed for 2 min with 100 μL of TSB at 1.0 dyn cm^{-2}. For the biofilm of *S. aureus* was made a coating with 100 μL of 10 μg mL^{-1} fibronectin for 2 min at 1 dyn cm^{-2}. The fibronectin binding was performed for 1 h without flow. After priming, fibronectin was aspirated from the output wells and replaced with 100 μL of fresh overnight cultures diluted to an OD600 of 0.8. The channels were seeded by pumping from the output wells to the input wells at 2.0 dyn cm^{-2} for 5 s. Cells were then allowed to attach to the surface of the channels for 30 min at 37 °C. 2.0 mL of TSB was added to the input well and pumped at 1 dyn cm^{-2} for 12 h. We used two inlet wells, in the first we added to medium SPEP at a concentration of 200 U mL^{-1}. Bright-field images were taken at 40× magnification at 1-min intervals for a total of 720 time points.

2.7. Adhesion assays

Bacteria from 18 h cultures in BHI broth, grown in the absence of SPEP were further subcultured up to OD600 = 0.5 at 37 °C in BHI with or without 200 U mL^{-1} SPEP. HeLa cells, cultured in 24-well plates (Falcon) to obtain semi-confluent monolayers (1×10^5 cells/well) were then inoculated with 0.05 mL of bacterial suspensions in logarithmic-phase growth at an MOI of about 10 bacteria per cell. The adhesion assay was carried out by keeping cells and bacteria in contact for 1 h at 37 °C. 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 TSA agar to determine viable adherent bacteria. Adhesion efficiency was expressed as the percentage of the inoculated bacteria that adhered to HeLa cells.

2.8. Invasion assays

HeLa cells, cultured in 24-well plates, were infected with 0.05 mL of logarithmically grown bacteria in the presence or in the absence of SPEP as above described. The entry of *S. aureus* was tested by infecting cells for 1 h at 37 °C at an MOI of about 10 bacteria per cell. After incubation, 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 extracellular bacteria. Cells were then lysed by the addition of 0.025% Triton X-100 and plated on TSA to count viable intracellular bacteria. We further calculated invasion efficiency expressed as the percentage of adhered bacteria that were internalized.

2.9. Surface protein extraction and processing

The surface proteins were extracted according to the method of Tabouret with minor modifications [31]. Briefly, after centrifugation of 50 mL of each bacterial culture (OD600 = 0.6), pellets were washed twice in PBS and then suspended in 1 mL of PBS containing 1% SDS. Samples were incubated at 37 °C for 15 min and after centrifugation the supernatants were collected and used for SDS-PAGE and zymogram analyses. The protein content in the samples was determined by the Bradford procedure [32].

2.10. SDS-PAGE and zymogram

SDS-PAGE was carried out by standard methods [32] with an SDS-polyacrylamide separating gel (10% acrylamide, pH 8.8) and constant voltage (180 V) at room temperature. Following electrophoresis proteins were stained with Coomassie Brilliant Blue (Bio-Rad). Renaturing SDS-PAGE was performed according to the methods of Lauderdale [33], with some modifications. SDS-polyacrylamide separating gel (10% acrylamide, pH 8.8) containing 0.2% (wt/*v*) lyophilized *Micrococcus luteus* cells provided by Sigma, was used to detect the lytic activities. After electrophoresis, the gels were soaked (2 times, 15 min) in distilled water at room temperature. The gels were then transferred into the renaturing buffer (50 mM Tris–HCl pH 8.0 containing 1% Triton X-100) and shaken at 60 rpm for 2 h at 37 °C to allow renaturation. The renatured autolysins appeared as clear translucent bands on opaque background. For each experiment, two gels were prepared from the same stock solution and electrophoresed in the same apparatus at the same time. No difference in the migration of the standards due to the presence of *M. luteus* cells in the gel was noted.

2.11. In situ digestion

The Coomassie blue-stained spots of interest were excised from the gel and washed with acetonitrile (ACN) first and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubation with 10 mM DTT for 45 min at 56 °C. Cysteines were alkylated by treatment with 5 mM iodoacetamide for 15 min at room temperature in the dark. Gel particles were then washed with ammonium bicarbonate and ACN. Tryptic digestion was carried out using 12.5 ng μL^{-1} of enzyme in 50 mM ammonium bicarbonate pH 8.5 at 4 °C for 4 h. The buffer solution was then removed and a new aliquot of the enzyme/buffer solution was added for 18 h at 37 °C. A minimum reaction volume, enough for the complete rehydration of the gel was used. Peptides were then extracted washing the gel particles with 20 mM ammonium bicarbonate and 0.1% TFA in 50% ACN at room temperature and then lyophilized.

2.12. MALDI-TOF mass spectrometry

Positive Reflectron MALDI spectra were recorded on a Voyager DE STR instrument (Applied Biosystems, Framingham, MA). The MALDI matrix was prepared by dissolving 10 mg of alpha cyano in 1 mL of acetonitrile/water (90:10 *v*/*v*). Typically, 1 μL of matrix was applied to the metallic sample plate and 1 μL of analyte was then added. Acceleration and reflector voltages were set up as follow: target voltage at 20 kV, first grid at 95% of target voltage, delayed extraction at 600 ns to obtain the best signal-to-noise ratios and the best possible isotopic resolution with multipoint external calibration using peptide mixture purchased from Applied Biosystems. Each spectrum represents the sum of 1500 laser pulses from randomly chosen spots per sample position. Raw data were analyzed using the computer software provided by the manufacturers and are reported as monoisotopic masses.

2.13. NanoLC mass spectrometry

When necessary, peptide mixtures were analyzed by LCMS/MS using a 4000Q-Trap (Applied Biosystems) coupled to an 1100 nanoHPLC system (Agilent Technologies). The mixture was loaded on an Agilent reverse-phase pre-column cartridge (Zorbax 300 SB-C18, 5 × 0.3 mm, 5 m) at 10 L min^{-1} (A solvent 0.1% formic acid, loading time 5 min). Peptides were separated on an Agilent reverse-phase column (Zorbax 300 SB-C18, 150 mm × 75 m, 3.5 m), at a flow rate of 0.3 L min^{-1} with a 0%–65% linear gradient in 60 min (A solvent 0.1% formic acid, 2% ACN in MQ water; B solvent 0.1% formic acid, 2% MQ water in ACN). Nanospray source was used at 2.5 kV with liquid coupling, with a declustering potential of 20 V, using an uncoated silica tip from New Objectives (O.D. 150 μm, I.D. 20 μm, T.D. 10 μm). Data were acquired in Information Dependent Acquisition (IDA) mode, in which a full scan mass spectrum was followed by MS/MS of the 5 most abundant ions (2 s each). In particular, spectra acquisition of MS–MS analysis was based on a survey Enhanced MS Scan (EMS) from 400 *m*/*z* to 1400 *m*/*z* at 4000 amu s^{-1}. This scan mode was followed by an Enhanced Resolution experiment (ER) for the five most intense ions and then MS2 spectra (EPI) were acquired using the best collision energy calculated on the bases of *m*/*z* values and charge state (rolling collision energy) from 100 *m*/*z* to 1400 *m*/*z* at 4000 amu s^{-1}. Data were acquired and processed using Analyst software (Applied Biosystems).

2.14. MASCOT analysis

Spectral data were analyzed using Analyst software (version 1.4.1) and MS–MS centroid peak lists were generated using the MASCOT.dll script (version 1.6b9). MS–MS centroid peaks were threshold at 0.1 96% of the base peak. MS/MS spectra having less than 10 peaks were rejected. MS/MS spectra were searched against NCBInr database using the licensed version of Mascot 2.1 version (Matrix Science), after converting the acquired MS–MS spectra in Mascot generic file format. The Mascot search parameters were: taxonomy *S. aureus*; allowed number of missed cleavages 2; enzyme trypsin; variable post-translational modifications, methionine oxidation, pyroglu N-term Q; peptide tolerance 200 ppm and MS/MS tolerance 0.5 Da; peptide charge, from +2 to +3 and top 20 protein entries. Spectra with a MASCOT score <25 having low quality were rejected. The score used to evaluate quality of matches for MS–MS data was higher than 30.

3. Results

3.1. Identification of genes involved in adhesion and correlation with biofilm production in S. aureus strains

In staphylococci bacterial adhesion and biofilm formation depend on a complex interplay of adhesins comprising fibrinogen binding proteins (FnBP-A/B and ClfA), sialoprotein binding proteins (SdrC and SdrD), extracellular matrix binding protein (SasG), biofilm associated protein (Bap), proteins involved in PIA synthesis (icaADBC), autolysins (Alt), etc. [19]. The four staphylococcal strains here considered, 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. From a genetic point of view, the four *S. aureus* strains are identical for the presence of *ica* operon and adhesion genes, excepting the only methicillin resistant *S. aureus* (MRSA), BAA1556 strain that did not possess *icaR* and *icaD* genes.

The biofilm-forming ability of *S. aureus* strains was tested by quantitative assay. They showed different capabilities to form biofilm that can be schematized as reported: three strains were strong

Table 2
Genotype characterization of staphylococcal strains.

Putative function of encoded protein	*S. aureus*			
	ATCC 6538P (DSMZ 346)	ATCC 25923 (DSMZ 1104)	ATCC 12598 (DSMZ 20372)	ATCC BAA1556 (USA 300 FPR3757)
Biofilm production	Strong	Strong	Weak	Strong
N-Acetylglucosaminyltransferase involved in PIA synthesis (*ica* locus)	+	+	+	$icaR^{-}$, $icaD^{-}$
Autolysin gene involved in initial adhesion (*atl*)	+	+	+	+
Fibronectin adhesin (*fnbA*)	+	+	+	+
Putative adhesin with unknown ligands (*sdrC*)	+	+	+	+
Fibrinogen adhesin (*clfA*)	+	+	+	+
Involved in biofilm formation in *S. aureus* bovine mastitis isolates (*bap*)	−	−	−	−
Adherence to desquamated nasal epithelial cells (*sasG*)	−	−	−	−

biofilm producers with biofilm amount higher than 1.6 OD at 590 nm, while ATCC 12598 is a medium biofilm producer (0.35 ± 0.05) according to Cafiso and co-workers [34].

3.2. SPEP treatment hinders biofilm formation of S. aureus strains

Previous experiments showed that SPEP did not affect the planktonic growth of *S. aureus*, up to a concentration of 1000 U mL^{-1} (data not shown). Preliminary experiments were assessed to define the best concentration of esoprotease to adopt for anti-biofilm response. Fig. 1 showed the dose-dependent effect on biofilm formation of 6538P strain used as a reference, starting from a concentration of 800 U mL^{-1}. Also at lower concentration used (0.39 U mL^{-1}) SPEP is efficient to remove biofilm (percentage of residual biofilm 38.7 ± 11.8). The concentration of SPEP adopted in the present study for further experiments was 200 U mL^{-1} because it assures a quite complete disaggregation of biofilm (less than 5% of residual biofilm).

Results of SPEP effect on biofilm formation of four *S. aureus* strains are summarized in Table 3. The effect of SPEP was related to the biofilm formation capacity of each staphylococcal strain here considered. SPEP action seemed to be proportional to the ability to form biofilm of each tested strain. Moreover, the best performance was obtained on 6538P strain.

The effect of SPEP on *S. aureus* 6538P preformed biofilm was also tested. Results show a significant reduction in the absorbance of the treated samples demonstrating that SPEP is also extremely effective in dispersal of *S. aureus* preformed biofilm (absorbance at 590 nm: control 2.47 ± 0.27; SPEP-treated 0.12 ± 0.03). This result suggests that SPEP action is not restricted to initial bacterial attachment on abiotic surface but is also effective on mature biofilm.

Effect of SPEP treatment on biofilm formation was also evaluated on BioFlux system. BioFlux technology permits to acquire sequential bright-field images of a developing biofilm. The BioFlux system is a microfluidics device that precisely controls the flow of growth medium between two interconnected wells of a microtiter plate. By positioning the channel connecting the two wells over a window accessible for viewing by microscopy, biofilm growth can be monitored in a time-course assay in which images are collected at 1-min intervals. Bacterial strains used for this experiments were MSSA 6538P and MRSA BAA1556. As shown in the video compilations of the collected images (see Supplemental videos) and in some selected images reported in Fig. 2, growth of two bacterial strains in this system, in the absence of SPEP (bottom lanes of each panel), revealed an initial rapid growth of the bacteria, resulting in a confluent "lawn" of cells that was followed by a period of detachment (after 8 h for 6538P and 4 h for BAA1556). For both strains, the top lanes contained bacteria grown in the presence of SPEP. SPEP clearly impaired the biofilm formation confirming results obtained in static system.

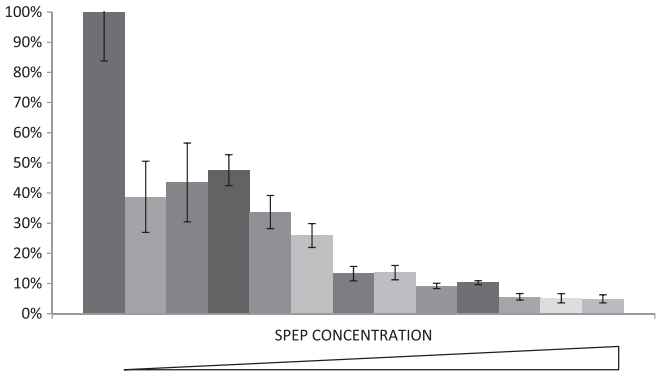

Fig. 1. A dose-dependent effect on biofilm formation of *S. aureus* 6538P in the presence of scalar concentration of SPEP (starting from 800 U mL^{-1}). Biofilm was expressed as the percentage of residual biofilm formation in comparison with untreated sample (100%, first row). Results are representative of three independent experiments.

Table 3
Effect of SPEP treatment on staphylococcal biofilm formation.

	Biofilm formation	
	Control	SPEP-treated
6538P	1.64 ± 0.15	0.086 ± 0.015
25923	1.68 ± 0.23	0.63 ± 0.12
12598	0.35 ± 0.05	0.21 ± 0.03
BAA1556	2.15 ± 0.08	0.47 ± 0.04

Based on the 590 nm OD absorbance produced by *S. aureus* strains. Data represent the mean ± SD of three independent experiments.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2013.05.003.

3.3. SPEP treatment affects adhesion and invasion efficiency of S. aureus 6538P and BAA1556

To evaluate the action of SPEP on the capability to adhere and invade to human cells an MSSA (6538P) and an MSRA (BAA1556) strain were used. Table 4 showed the adhesion and invasion efficiency of SPEP-treated and untreated bacteria. Adhesion was defined as the ratio between the number of bacteria adherent on HeLa cells and CFU of the inoculum. Invasion was obtained as the ratio between the number of internalized bacteria counted after lysis of gentamicin treated HeLa cells and CFU of the inoculum. Our results showed that the adhesion efficiency of 6538P was unaffected by enzymatic incubation (untreated: 2.75 ± 0.45; SPEP-treated: 2.80 ± 1.80) while as regards BAA1556 the adhesion efficiency was partially upset by the SPEP (untreated: 4.94 ± 0.97; SPEP-treated: 2.82 ± 1.46). About the invasion, expressed as the percent of the adhered bacteria which invaded HeLa cells, our data showed that about 50% of the BAA1556 adhering to HeLa cells invaded them (adhesion: 4.94 ± 0.97; invasion: 2.5 ± 0.79). On the contrary the percentage of 6538P that invaded HeLa cells was very low (0.7×10^{-2} of initial inoculum). The invasion efficiency was drastically reduced in both strains (about 200-fold for the 6538P and 3000-fold for the BAA1556) following SPEP treatment.

3.4. SDS-PAGE and zymogram analyses of surface proteins in SPEP-treated and untreated S. aureus

Cell surface protein samples from SPEP-treated and untreated *S. aureus* cultures were simultaneously analyzed by SDS-PAGE and zymogram assays. Fig. 3A shows the SDS-electrophoretic profiles of the protein mixtures obtained from surface protein extraction following colloidal Coomassie blue staining. Several discrete protein bands corresponding to the surface proteins extracted from untreated *S. aureus* cells were observed in the control lane. This profile was compared to the protein pattern obtained after SPEP treatment. Various, specific protein bands detected in the untreated *S. aureus* protein profiles either disappeared or their intensity was drastically reduced after enzyme incubation. This effect was clearly visible for all bacterial strains analyzed.

Fig. 3B shows the zymogram profiles of SPEP-treated and untreated surface proteins extracted from *S. aureus* strains. In the separation range considered, various autolysin bands were detected for both treated and untreated samples whereas SPEP treatment led to the disappearance or to a reduction of intensity of several autolysin bands. Comparative experiments were carried out in the same conditions using proteinase K as a proteolytic

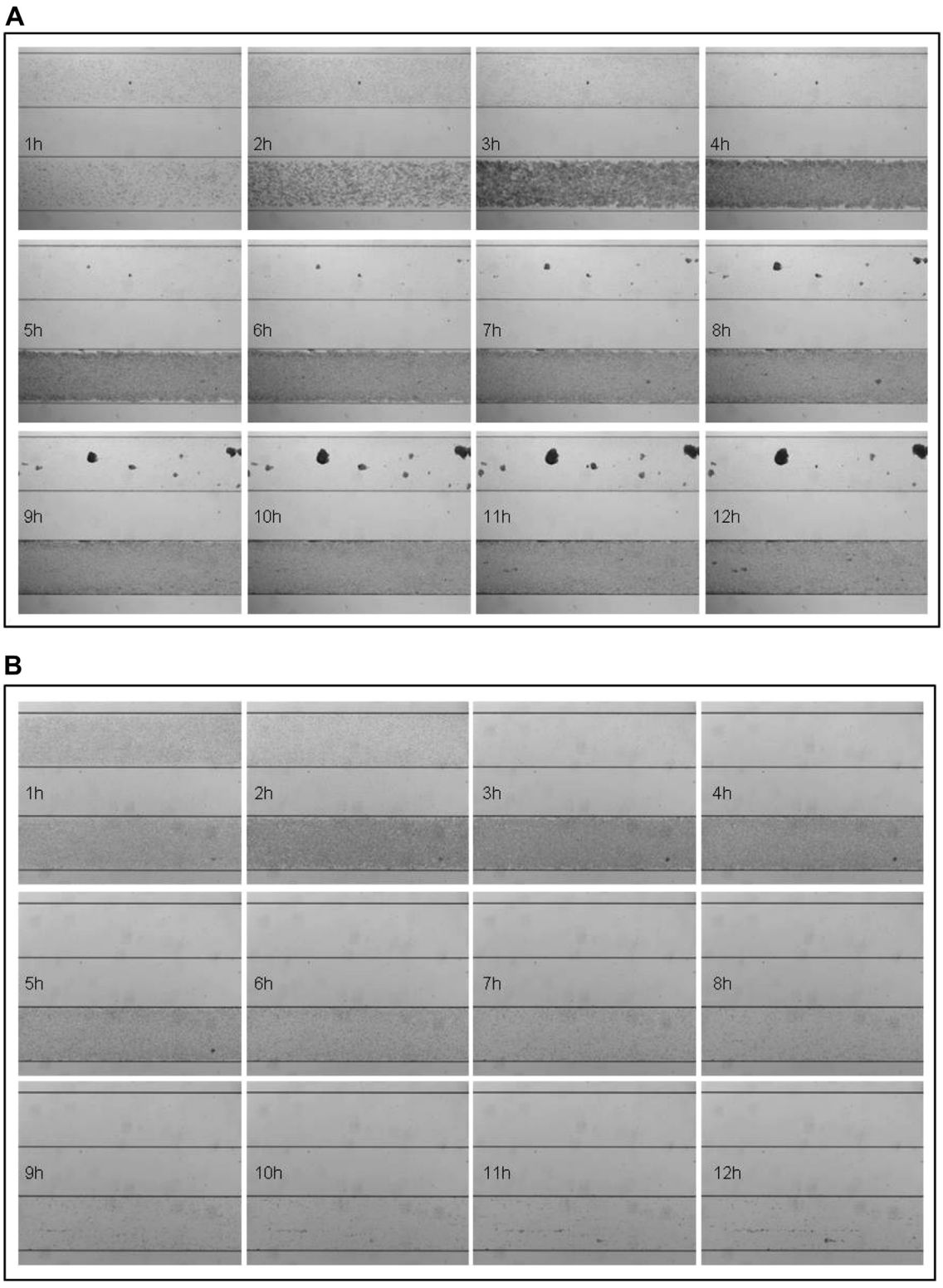

Fig. 2. Biofilm formation and development of *S. aureus* 6538P (A) and *S. aureus* BAA1556 (B) in a BioFlux system. Each image contains two channel: top channel was SPEP-treated sample and bottom channel was the control one. Bright-field microscopic images were collected at 1-min intervals. The images presented were taken from the complete set of 720 images (see Supplementary video S1 for a video compilation of these images) taken at 40× magnification. For complete video compilation of this experiment, see Video S1.

agent on *S. aureus* 6538P. Similarly to SPEP, proteinase K incubation totally abolished biofilm formation. However, when analyzed by SDS-PAGE, all the surface protein bands disappeared in the sample treated with proteinase K, suggesting a non-specific and indiscriminate effect of this protease (Fig. 3A, top panel, lane P).

3.5. Identification of S. aureus BAA1556 proteins by mass spectrometry analysis

The protein bands occurring in the untreated *S. aureus* protein profile and disappearing upon SPEP treatment were selected for

Table 4
Adhesion and invasion capabilities of SPEP-treated and untreated *S. aureus*.

	Untreated		SPEP-treated	
	Adhesion[a]	Invasion[b]	Adhesion[a]	Invasion[b]
6538P	2.75 ± 0.45	$0.7 \times 10^{-2} \pm 0.1 \times 10^{-2}$	2.80 ± 1.80	$0.3 \times 10^{-4} \pm 0.0 \times 10^{-4}$
BAA1556	4.94 ± 0.97	2.5 ± 0.79	2.82 ± 1.46	$0.7 \times 10^{-3} \pm 0.1 \times 10^{-3}$

[a] Adhesion is expressed as the percentage of the initial inoculum of bacteria that adhered to HeLa cells 1 h post-infection at 37 °C.
[b] Invasion efficiency is expressed as the percentage of adhered bacteria that were gentamicin resistant 1 h post-infection. Data represent the mean ± SD of three independent experiments.

further analyses. We have been already identified surface proteins of 6538P strain impaired by SPEP treatment [28]. In this work we chose to analyze the surface proteins of MRSA strain BAA1556. In Fig. 4 the proteins that disappeared following SPEP incubation were marked with black dots. These proteins were selectively excised from the gel and identified by mass spectrometric analyses. Gel slices from the corresponding areas in the protein profile of treated BAA1556 strain were also selected and used as a control in the identification procedure. All samples were reduced, alkylated and digested *in situ* with trypsin; an aliquot of the resulting peptide mixtures were directly analyzed by MALDI-TOF mass spectrometry. Spectral data were then used to search a non-redundant protein sequence database using an in house version of the MASCOT software. When this procedure did not provide confident identification of the proteins, the remaining portion of the peptide mixtures were analyzed by LCMS/MS. Proteins identified by mass spectrometric analyses are listed in Table 5. The results obtained indicated that SPEP treatment led to the disappearance of specific proteins, including some surface proteins known to be involved in adhesion and invasion of host cells, such as Atl, SdrD, SsA2 and Sbi, homolog to SpA and component of MSCRAMMs molecules. Remarkably, SPEP treatment also affected selected proteins with a function in the cytoplasmic compartment related to energy production and carbohydrate metabolism and particularly involved in the glycolytic pathway. Elongation factor EF-Tu, elongation factor EF-G and the dihydrolipoamide acetyltransferase component of the pyruvate dehydrogenase complex [35,36] are other examples of these multifunctional factors belonging to energetic metabolic pathways but also involved in adhesion to extracellular matrix and invasion of host cells. These proteins have been shown to be present in the cell wall of pathogenic bacteria endowed with adhesion and invasion capabilities and able to induce proinflammatory response [37].

4. Discussion

The capacity of bacterial cells to adhere, grow in biofilm and invade eukaryotic cells is a crucial step for the colonization and for their survival in the environment. It depends on wide array of

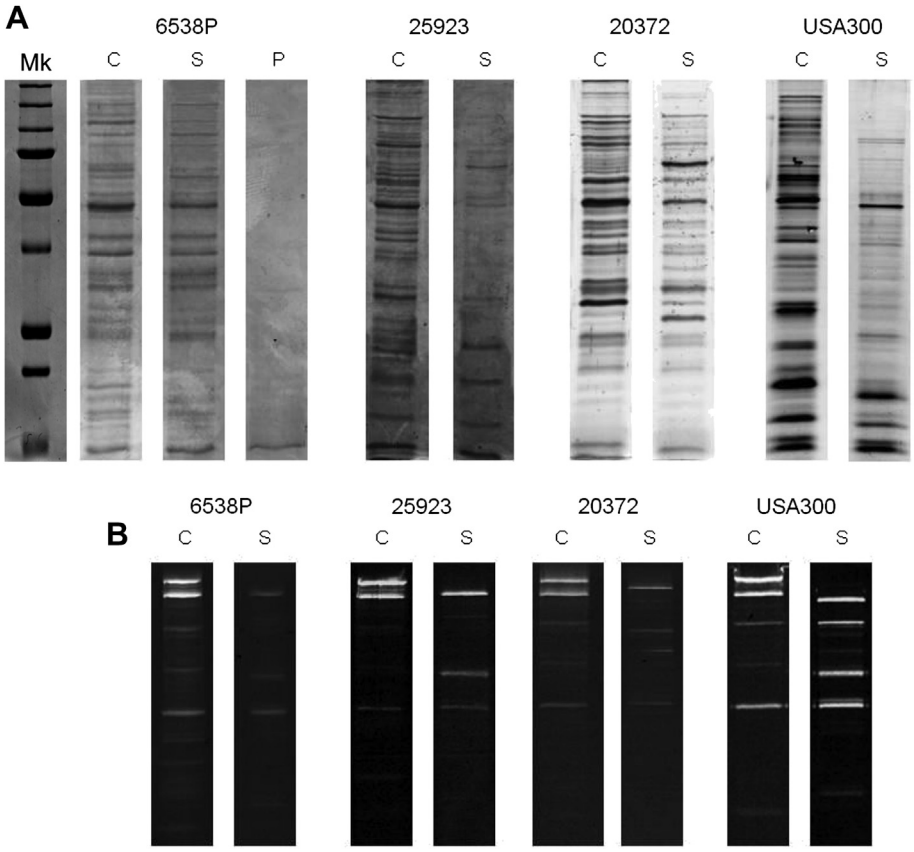

Fig. 3. SDS-PAGE and zymogram analyses of surface proteins of four *S. aureus* strains. Crude cell envelope SDS extracts from SPEP-treated and untreated (control) *S. aureus* analyzed by (A) SDS-PAGE and (B) zymogram assays. Mk: precision Plus prestained marker; Biorad (range 250–10 kDa); C: control; S: SPEP-treated samples; P: proteinase K-treated sample. Autolysins formed translucent areas in the zymogram.

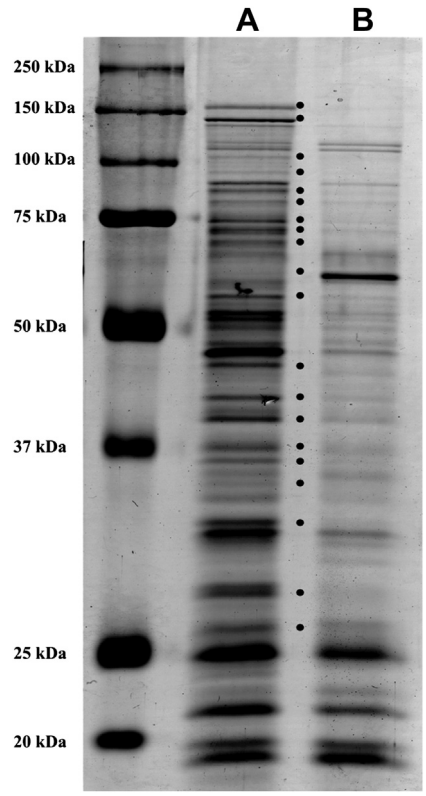

Fig. 4. Crude cell envelope SDS extracts from (A) untreated, (B) SPEP-treated *S. aureus* BAA-1556 cells analyzed by SDS-PAGE. Protein bands indicated with black dots were present in the untreated samples and disappeared or drastically reduced following enzymatic treatment, were identified by mass spectrometric analysis. The corresponding gel slices from treated sample lanes were also submitted to the identification procedure as a control.

secreted and cell surface-associated virulence factors, including proteins that promote adhesion to damaged tissue and to the surface of host cells, and that bind proteins in blood to help evade immune responses [38–41]. Different surface proteins of *S. aureus* provide ingenious strategies for bacterial escape from the host immune response [42]. For this reason, bacterial surface proteins constitute novel putative candidates for the development of protective polyvalent vaccines because they are critically important in determining the success of bacterial strains in their competition for survival. Actually, most of *S. aureus* involved in the pathogenesis are resistant to multiple antibiotics; these strains are known as methicillin resistance *S. aureus* (MRSA). In this study a clone of MRSA USA 300, the major source of community-acquired infections in the USA, Canada, and Europe [43] was also used.

Table 5
S. aureus BAA1556 surface proteins disappearing following SPEP treatment identified by MS analysis.

Protein	MW	Swissprot
Adhesion and cell wall		
Serine–aspartate repeat-containing protein D (sdrD)	149,416	Q2FJ78
Bifunctional autolysin (Atl)	137,339	P0C5Z8
IgG-binding protein (Sbi)	50,012	Q931F4
Staphylococcal secretory antigen ssaA2	29,366	Q5HDQ9
Translation		
Elongation factor G (EF-G)	76,849	Q2FJ93
Glutamyl-tRNAGln amidotransferase subunit A (Glu-ADT subunit A)	52,939	Q2FFJ5
Elongation factor Tu (EF-Tu)	43,104	Q2FJ92
Energy and metabolism		
Carbamoyl-phosphate synthase large chain	117,554	Q2FHN5
Aspartyl-tRNA synthetase (AspRS)	66,728	Q2FG97
Pyruvate kinase (PK)	63,291	Q2FG40
Transketolase (tkt)	62,250	Q5HG77
Malate dehydrogenase (MQO)	55,999	Q2FDQ3
ATP synthase F1 sector subunit alpha	54,607	P63676
Dihydrolipoamide dehydrogenase component of PDH (pdhC)	49,592	P0A0E6
Dihydrolipoyllysine-residue acetyltransferase component of PDH	46,468	Q5HGY9
Putative NADH dehydrogenase	44,362	Q6GIE7
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH 1)	36,372	P0A036
Pyruvate dehydrogenase E1 component beta subunit	35,224	P0A0A1
Triosephosphate isomerase	27,416	Q2FIL9

The study of new strategies aiming at controlling of virulence factors can revert pathogens to a phenotype susceptible to immune defenses and antibacterials without increasing their drug resistance. In the search for new treatments specifically addressed against staphylococcal virulence factors, such as the ability to invade host cells and to form biofilm, we investigated the effect of SPEP on several *S. aureus* properties associated to the virulent phenotype. The recombinant form of SPEP is actually widely used as an anti-inflammatory drug and as a mucoactive agent [24,44]. The tertiary structure of this protein contains two different domains: a catalytic domain with the zinc atom essential for the proteolytic activity and an uncharacterized domain, whose function is still unknown. This latter could potentially be involved in the modulation of virulent phenotype [22].

Preliminarily we examined the genetic background of the strains here used. The only genetic difference is represented by *ica* locus partially lacking in the MRSA strain BAA1556. Although, several studies determined that mutations in the *ica* locus in multiple *S. aureus* strains do not impair biofilm capacity, revealing a second *ica*-independent mechanism of biofilm formation. In addition, examination of MRSA strains indicates that these isolates predominantly form the *ica*-independent biofilm [45].

The effect of SPEP on *S. aureus* biofilm formation was compared with the action of a known and well-characterized protease, the proteinase K. Also proteinase K treatment completely inhibited biofilm formation. However, following proteinase K incubation, surface proteins of *S. aureus* completely disappeared, thus indicating a random and unspecific action of proteinase K, leading to a putative proteolytic degradation of cell exposed proteins. Conversely, SPEP treatment showed a specific and reproducible action on bacterial cells, leading to the disappearance of discrete protein bands in the surface protein pattern. Thus SPEP seems to exert its action by either removing or negatively regulating specific bacterial surface proteins. This effect was observed for all strains here analyzed. Furthermore, previously experiments on *Listeria monocytogenes* provided the same results [25]. Various evidences obtained in the present study demonstrate that *in vitro* treatment of *S. aureus* cells with non-cytotoxic, non-bactericidal and non-bacteriostatic concentrations of the enzyme can negatively modulate the expression of the virulent phenotype. Preliminary experiments were carried out to assess the effect of SPEP on the growth rate of *S. aureus* ATCC 6538P. SPEP did not affect *S. aureus* duplication rate. In fact, bacterial growth curves were nearly superimposible both in the presence and in the absence of SPEP [28]. Furthermore, SPEP cytotoxicity was assessed on eukaryotic cells. Cell morphology, viability and proliferation of HeLa cells remained unaffected by SPEP treatment (data not shown).

While *S. aureus* has not traditionally been considered to be an intracellular pathogen, previous studies have revealed that it may be actively internalized by phagocytosis and are capable of intracellular survival in epithelial and endothelial cells [46]. Data reported showed that the MRSA strain possessed invasion capability 300-fold higher than MSSA strain. This feature, together with the presence of mobile genetic elements that carry genes encoding

superantigenic and other toxins, could also explain the marked persistence of this clone in the pathogenesis.

Nevertheless following SPEP treatment, the invasive efficiency of both MRSA and MSSA strains infecting HeLa cells were significantly reduced (about 200-fold for 6538P and 3000-fold for BAA1556).

Comparative proteomic investigations on SPEP treated and untreated *S. aureus* cells revealed a discrete number of surface proteins affected by the enzymatic treatment. Among these, some proteins like EF-Tu, EF-G and several glycolytic enzymes are involved in energy metabolism and localized in the cytosol. However, these proteins have also been reported to be immunogenic, localized on the cell wall and associated with the most invasive isolates [35,36]. Moreover, investigation of the surface subproteome of *L. monocytogenes* revealed a remarkably high number of proteins with a function in the cytoplasmic compartment [37]. A number of reports have also indicated that anchorless surface proteins perform a variety of functions that facilitate bacterial colonization, persistence and invasion of host tissues [47–49]. It is now widely accepted that proteins may fulfill several biological functions depending on the partners they transiently associate with. It descends that metabolic proteins are not only required for energy production but are also essential for efficient host cell invasion, indicating an alternative role for these proteins on the surface of bacterial cells.

Some members of the adhesion family, Atl, Sbi, EF-Tu, EF-G, and sdrD identified in this study deserve particular attention. Atl disappeared after SPEP treatment, confirming that SPEP modulates adhesins and autolysins also in *S. aureus*. Moreover, zymogram assays suggested an influence of SPEP on various proteins possessing lytic activity. Recently, a novel mechanism involved in staphylococcal internalization by host cells, which is mediated by the major autolysin/adhesins Atl in *S. aureus* has been discovered [50]. Sbi, a member of MSCRAMMS homologous to the immunoglobulin G-binding protein A (SpA), is a multifunctional bacterial protein which binds host complement components Factor H and C3 as well as IgG and b2-glycoprotein I and interferes with innate immune recognition. Sbi acts as a potent complement inhibitor, and inhibits alternative pathway-mediated lyses of rabbit erythrocytes by human serum [51,52].

SD-repeat containing protein D (SdrD), an MSCRAMM family surface protein, plays an important role in *S. aureus* adhesion and pathogenesis. In a very recent publication, the crystal structure of such domains of this protein has been elucidated and the ligand binding site of SdrD was characterized [53].

EF-Tu and EF-G belong to the so called 'moonlight' proteins, endowed *per se* with multiple functions that are not associated to gene fusions, splice variants or multiple proteolytic fragments [5]. EF-Tu was identified as a major cell wall associated component of many bacteria; among them *S. aureus* [36], *Lactobacillus johnsonii* [54], *Mycobacterium leprae* [55]; *Mycoplasma pneumoniae*, where it mediates fibronectin binding together with the pyruvate dehydrogenase E1 subunit [56,57]. In *L. monocytogenes* EF-Tu was identified together with EF-G. Recently, EF-Tu has been identified as a surface protein possessing the characteristics of an adhesion factor and showing the capacity to induce a proinflammatory response [58]. This study demonstrates that SPEP treatment selectively affects a discrete number of surface proteins clearly involved in fundamental mechanisms associated with bacterial virulence, such as adhesion, invasion and biofilm formation. A major question still remains to be answered, whether this action is due to the proteolytic function of SPEP directed towards very specific protein targets or to the triggering of specific signal transduction pathways elicited by SPEP negatively regulating protein expression. Data reported in this paper, together with our previous results [25,28], call attention to SPEP as a noncytotoxic natural compound effective as an anti-virulence drug against different Gram-positive pathogens. SPEP treatment seems to be addressed towards specific *S. aureus* determinants associated with bacterial virulence, hampering the attachment of bacterial cells to different surfaces moieties. It is important to stress that SPEP does not affect bacterial viability when used at the concentrations adopted in this work and at higher concentrations. One of the greatest problems in developing an effective antibacterial approach consists in the rapid appearance of mutants insensitive to the therapy. Traditional antibacterial compounds target fundamental processes needed for bacterial survival, increase mutants selection and diminish the efficacy of the therapy itself. Recent therapeutic strategies are addressed to bacterial virulence so as weaken bacteria without directly killing them. In this respect, SPEP could be developed as a novel anti-virulence tool that would hinder the entry of *S. aureus* into human tissues, and also impairs the ability of this pathogen to form biofilm on prostheses, catheters and medical devices.

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