Microbiological Research 216 (2018) 85–96

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

The exoproteome profiles of three *Staphylococcus saprophyticus* strains reveal diversity in protein secretion contents

Andrea Santana de Oliveira[a], Isabella Inês Rodrigues Rosa[a], Evandro Novaes[b], Lucas Silva de Oliveira[a], Lilian Cristiane Baeza[c], Clayton Luiz Borges[a], Lennart Marlinghaus[d], Célia Maria de Almeida Soares[a], Marcia Giambiagi-deMarval[e], Juliana Alves Parente-Rocha[a,*]

[a] *Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil*
[b] *Escola de Agronomia, Universidade Federal de Goiás, Goiânia, Goiás, Brazil*
[c] *Centro de Ciências Médicas e Farmacêuticas, Universidade Estadual do Oeste do Paraná, Cascavel, Brazil*
[d] *Department of Medical Microbiology, Ruhr-University, Bochum, Germany*
[e] *Laboratório de Microbiologia Molecular, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro – Rio de Janeiro, Rio de Janeiro, Brazil*

ARTICLE INFO

Keywords:
Staphylococcus saprophyticus
Exoproteome
Diversity
SsaA secretion
Proteases
Antigenic proteins

ABSTRACT

Staphylococcus saprophyticus is a gram-positive microorganism responsible for urinary tract infections (UTIs). Although some virulence factors are characterized, such as urease, autolysins, adhesins and hemagglutinins, large-scale proteomic studies have not been performed within this species. We performed the characterization of the exoproteome from three *S. saprophyticus* strains: the reference strain ATCC 15,305, a non-capsular strain 7108 and the 9325 strain containing a thick capsule which were cultured in BHI medium and culture supernatants were analysed by using mass spectrometry approach. We observed a core of 72 secreted proteins. In addition, it was possible to detect diversity in the protein profiles of the exoproteomes. Interestingly, strain 7108 presented no secretion of three antigenic proteins, including the classical SsaA antigen. In addition, the level of antigenic proteins secreted by strain 9325 was higher than in ATCC 15,305. This result was confirmed by Western blot analysis using anti-SsaA polyclonal antibodies, and no production/ secretion of SsaA was detected in strain 7108. Transcriptional data shows that 7108 strain produces transcripts encoding SsaA, suggesting post-transcriptional regulation occurs in this strain. Moreover, when compared with the other strains that were analyzed, it was possible to detect higher levels of proteases secreted by strain 7108 and higher levels of antigenic proteins and transglycosylases secreted by 9325 strain. The results reveal diversity in protein secretion among strains. This research is an important first step towards understanding the variability in *S. saprophyticus* exoproteome profile and could be significant in explaining differences among strains.

1. Introduction

Staphylococcus saprophyticus is a gram-positive bacterium that causes urinary tract infections (UTIs) (Gatermann et al., 1988). This species is the causative agent of 40% of UTIs in young women (Raz et al., 2005). The pathogenicity of *S. saprophyticus* is not completely known, but some virulence factors have been described. Surface proteins, such as Ssp protein and hemagglutinin, are described as being active in the bacteria attachment in the bladder (Gatermann et al., 1993). Urease has been known as a virulence factor in *S. saprophyticus*, contributing to urophatogenicity in rats (Gatermann et al., 1989). The inhibition of urease activity can delay *S. saprophyticus* growth in artificial urine medium (Loes et al., 2014). Another important virulence factor of *S. saprophyticus* is D-serine-deaminase protein (DSDA), since D-serine is encountered in urine where it acts as a bacteriostatic. A mutant for *dsda* gene, when exposed to the murine model, has presented attenuated virulence (Korte-Berwanger et al., 2013). Despite the prognosis in the majority of infections caused by *S. saprophyticus*, this bacterium can be seen to spread in the environment without difficulty. Furthermore, *S. saprophyticus* presents temporal persistence and can carry cassette resistance to other species, as the presence of erythromycin cassette resistances to ermC, msrA, msrB, mphC, and linA were found in 93% of the strains isolated in Brazil (Sousa et al., 2017).

In *Staphylococcus aureus*, the pathogenicity results from the synthesis of cell surface-associated proteins and extracellular proteins influencing a highly variable set of virulence factors (Kusch and Engelmann,

* Corresponding author.
E-mail address: juliana.parente@pq.cnpq.br (J.A. Parente-Rocha).

https://doi.org/10.1016/j.micres.2018.08.008
Received 4 May 2018; Received in revised form 7 August 2018; Accepted 21 August 2018
Available online 23 August 2018

2014). Proteins that are targeted for secretion by the classical pathway generally possess a signal peptide at the N-terminal extremity. There are seven known secretory systems in bacteria. They are known individually as a "Type Secretion System" (TSS), and each one secretes particular toxins and other important proteins for pathogens (Tseng et al., 2009). Non-classical pathways with unclear mechanisms also occurs in bacteria (Wang et al., 2016).

One aspect that can influence the ability of *S. aureus* to cause infection is the presence of capsular polysaccharide (CP). Strains containing CP presents enhance virulence and survival *in vivo* in mice model compared to non-capsular strain (Watts et al., 2005). *S. saprophyticus* strains can present CP but is not required to cause infection since capsular (such as ATCC 15,303 and 9325 strains) and non-capsular (such as 7108 strain) strains were isolated from patients and can be internalized into human bladder cells at similar rates (Szabados et al., 2008). One study performed using 236 *S. saprophyticus* strains osilated from patients demonstrated that capsule was presented in 1.3% of the strains. The absence of capsule is related to a hydrophobic profile and can confer adherence properties since surface adherent proteins, such as SdrI and UafA, are more exposed. The capsule confers a hydrophilic profile and absence of hemagglutination ability. The degradation of the capsule of 9325 strain turned the cells hydrophobic, suggesting the capsule is responsible for masking hydrophobic profile (Kleine et al., 2010). In counterpart, the capsule is associated to a resistant mechanism to shield its surface from recognition by the host immune system (O'Riordan and Lee, 2004). In the same way, the capsule can contribute inhibiting UafA-mediated adherence of *S. saprophyticus* to the T24 cell line (Kuroda et al., 2005) and can increase the resistant to complement-mediated opsonophagocytic killing by human neutrophils (Park et al., 2010).

Other important aspect that can contribute to the ability to establishment of infection in the human host in the presence of genes encoding virulence factors. Several known virulence factors have been tracked in *S. saprophyticus* strains, such as: *ure* (encoding urease), *sdrI* (encoding collagen-binding protein), *ssp* (encoding surface associated lipase), *dsd* (encoding D-serine deaminase), *capD* (encoding UDP-GlcNAc 4,6-dehydratase), *aas* and *uafA* (encoding surface proteins) and the regulatory genes *agr*, *sarA* and *rot*. Interestingly, studies performed with 236 *S. saprophyticus* strains (including ATCC 15,305, 7108 and 9325 strains) demonstrated that 100% of the strains possess genes encoding urease, UafA, DsdA, Aas and regulatory genes *agr, sarA* and *rot*, suggesting these genes are required to survival and/or infection. On the other hand, gene encoding SdrI is detected in 10% of strains isolated from patients and gene encoding Ssp in detected in around 87% of the these strains (7108 possess both genes and ATCC 15,305 and 9325 are negative for both genes) (Kleine et al., 2010).

Although a large amount of proteomic data from the *Staphylococcus* species is available (Atshan et al., 2015; Carvalhais et al., 2015; Bonar et al., 2016), no large-scale proteomic study has been performed with *S. saprophyticus*. Added to this, the virulence factors described for *S. saprophyticus* are mostly associated with cell wall and just urease protein is a secreted protein characterized in this specie as virulence factor. In this study, we performed identification and differential expression analyses of secreted proteins profiles from three *S. saprophyticus* strains presenting phenotypic differences in the capsule: ATCC 15,305, 7108 and 9325 The 9325 strain possess a thick capsule while ATCC 15,305 possess a lower capsule and 7108 is non-capsular strain. It was possible to identify proteins secreted in all isolates, as well as specifically-secreted proteins. In order to validate the proteomic analyses, we selected the SsaA protein, an antigenic protein whose homologous protein in *S. epidermidis* was detected in high levels in patients presenting endocarditis (Lang et al., 2000). Western blot analysis was performed and the results corroborate the proteomic data. The comparative analysis between the *S. saprophyticus* exoproteomes will contribute to the elucidation of the mechanisms that can be used by different strains to promote pathogenesis in the genitourinary tract.

2. Material and methods

2.1. Ethics statement

This study was approved by the Ethics in Research Committee of the "Universidade Federal de Goiás" (protocol Nº047/13). The animal experiments were conducted in accordance with legislation for the protection of animals used for scientific purposes (EU Directive 2010/63).

2.2. S. saprophyticus strains and culture conditions

Staphylococcus saprophyticus strains ATCC 15,305, 7108 and 9325 were used in this study. These strains were previously phenotypically characterized and they differ in the presence of capsule (ATCC 15,305 possess capsule, 7108 is non-capsular and 9325 possess a thick capsule) and in the presence of virulence factors (Kleine et al., 2010; Park et al., 2010). *S. saprophyticus* cells were cultured in BHI medium (Sigma-Aldrich, St. Louis, MO, USA) and stored at -80 °C in 50% (v/v) glycerol. In order to obtain the exoproteome from the isolates, a single *S. saprophyticus* colony of each strain was pre-incubated separately in BHI medium until the stationary phase (after 18 h) with shaking at 36 °C. The *S. saprophyticus* cells were harvested by centrifugation at 4000 *g* for 10 min, washed with PBS and transferred to the same volume of a fresh BHI medium for 1, 3 and 6 h at 100 *g* and 36 °C to establish the best time point for proteomic analysis. The supernatant was collected after this period of incubation. Proteomic analyses were performed following 3 h of incubation in BHI medium after the stationary phase was obtained.

2.3. Obtaining the secreted proteins by S. saprophyticus

The cultures were centrifuged at 4000 *g* for 10 min, and the supernatant was filtered through 0.45-µm and 0.22-µm filters (Millipore®, Bedford, MA, USA). Trichloroacetic acid (TCA) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 10% (w/v) was added to the supernatant, and protein precipitation was performed for 16 h at 4 °C. A protein pellet was obtained by centrifuging the supernatant for 1 h at 5000 *g* and 4 °C. The pellet was washed 3 times with cold acetone (Sigma-Aldrich, St. Louis, MO, USA), resuspended in 50 mM ammonium bicarbonate buffer (Sigma-Aldrich, St. Louis, MO, USA), pH 8.5 and concentrated in a 3-kDa disposable Amicon ultrafilter (Millipore®, Bedford, MA, USA).

2.4. Analysis of S. saprophyticus cell growth and viability

The *S. saprophyticus* cell growth was monitored by spectrophotometry SpectraMax Paradigm (Molecular Devices, Lagerhausstrasse, Austria) in 660 nm wavelength. The experiment was performed with the three selected strains in duplicate. Standard error of the mean was obtained. Cells presenting optical density (OD) higher than 1.0 were diluted prior measuring of OD. The cells viability experiment was performed by using flow cytometry with propidium iodide to label dead cells, as previously described (Grossklaus et al., 2013). Control dead cells were obtained by boiling the samples during 10 min. A total of 1 mL of the culture in the same condition used for proteomic analysis and control dead cells were incubated with 1 µg of propidium iodide during 10 minutos before flow cytometry analysis. A minimum of 5000 cells per sample was acquired and the instrument used was Guava® easyCyte (Merck Millipore, Darmstadt, Germany).

2.5. Analysis of culture supernatant by polymerase chain reaction (PCR)

The PCR analysis of the culture supernatant was performed using genomic DNA as control. Genomic DNA was extracted after cell lysis in Bead Beater equipment (Biospec, Bartlesville, OK, USA) with glass beads (Sigma-Aldrich, St. Louis, MO, USA) using a standard method (Sambrook and Russel, 2001). The PCR reaction was performed using

Green Taq Master Mix (Promega, Madison, WI, USA), with 2 µL of concentrated supernatant culture or genomic DNA and oligonucleotides to amplify a 189-bp fragment of the *rpsa* gene (accession number BAE18415.1) encoding the 30S ribosomal protein S1. The oligonucleotides used were 30S sense: 5′ GTCGTAAAGCAGTAGAGGCATT 3′ and 30S antisense: 5′ AAACGTGAACAAGTCCATCAAC 3′. The reaction was performed with 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. The PCR amplicons were visualized in a 1% agarose gel stained with Gel Red (Biotium, Hayward, CA, USA). The PCR sensitivity was evaluated using genomic DNA as a template at concentrations of 50 ng to 1 pg.

2.6. Digestion of protein extracts for nano-ESI-UPLC-MS^E acquisition

Enzymatic digestion of proteins was processed according as described (Murad et al., 2011; Murad and Rech, 2012) with some modifications. In brief, a total of 500 µg of protein extract was used for trypsin digestion. The samples were treated with RapiGEST™ SF Surfactant (0.2% v/v) (Waters, Milford, MA, USA) in a dry bath at 80 °C for 15 min. The reduction of disulfide bonds was performed with 100 mM dithiothreitol (DTT) (GE Healthcare, Piscataway, NJ, USA) at 60 °C for 30 min, and alkylation of cysteine with 300 mM iodocetamide (GE Healthcare, Piscataway, NJ, USA) for 30 min at room temperature. The proteins were subsequently digested with 5 U of trypsin (Promega, Madison, WI, USA) at 37 °C in a dry bath for 16 h. Afterwards, the samples were treated with 5% trifluoracetic acid solution (Sigma-Aldrich, St. Louis, MO, USA) incubated for 90 min at 37 °C in a dry bath, and centrifuged at 18,000 *g* at 4 °C for 30 min. The supernatants were dried in a speed vacuum (Eppendorf, Hamburg, Germany). All obtained peptides were suspended in 80 µL of a solution containing 20 mM of ammonium formiate and 150 fmol/µL of PHB (Rabbit Phosphorylase B) (Waters Corporation, Manchester, UK) as internal standard.

Nanoscale LC separation of tryptic peptides was performed using a ACQUITY UPLC® M-Class system (Waters Corporation, USA), as described (de Oliveira et al., 2018). The lock mass was used for calibration of the apparatus, using GFP ([Glu1]-Fibrinopeptide B human (Sigma-Aldrich, St. Louis, MO, USA). Mass spectrometry analysis was performed on a Synapt G1 MS™ (Waters, USA). Samples were analyzed from three replicates.

2.7. Data processing and protein identification

The mass spectrometer data obtained were processed using the ProteinLynx Global Server version 3.0.2 (Waters, Manchester, UK). The data were subjected to automatic background subtraction, deisotoping and charge state deconvolution. The processed spectra were searched against protein sequences from *S. saprophyticus* (http://www.uniprot.proteomes/) and against a databank from reverse sequences. This predicted database from reversed proteins is used to calculate false positive rate. The mass error tolerance for peptide identification was under 50 ppm. The identification of protein was allowed with a maximum 4% false discovery rate in at least three technical replicate injections. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2016) *via* the PRIDE partner repository (Project number PXD008643).

2.8. Statistical analysis of proteomic data

The n-gram of each protein, from each *S. saprophyticus* strain and replicate, was used as a measure of protein expression. The n-gram data were put into the R software and combined into a matrix, where rows indicated the proteins, and columns the samples (strains x replicates). Expression data were $\log_2$ transformed and quantile normalized with the limma package (Ritchie et al., 2015) using the normalizeBetweenArrays function. Proteins with excessive missing data (more than six of the nine total samples) were subtracted from the analyses. Differential expression analyses between the three *S. saprophyticus* strains were performed with an empirical Bayes method implemented in the limma package (Phipson et al., 2016). Proteins were declared differentially expressed using a threshold of 0.05 false discovery.

Uniprot (http://www.uniprot.org), as well as Pedant in the MIPS (http://mips.helmholtz-muenchen.de/funcatDB/) database, were used for functional classification. The NCBI database was employed for annotation of uncharacterized proteins and for re-annotation of proteins that presented annotation errors (https://www.ncbi.nlm.nih.gov/).

2.9. Cloning and heterologous expression of SsaA from S. saprophyticus

The complete gene sequence encoding the SsaA protein (*ssaA*) was obtained in the NCBI database (accession number BAE17758.1) and used to design oligonucleotides for the In-Fusion HD Cloning Kit (Clontech) in the online Tool (http://www.clontech.com/US/Support/xxclt_onlineToolsLoad.jsp?citemId=https://www.takara-bio.co.jp/infusion_primer/infusion_primer_form.php&section=16260&xxheight=1800). The restriction site for *Eco*RI was used to clone the gene *ssaA* in the pGEX-4T-3 expression vector. The oligonucleotides were synthesized (IDT-Integrated DNA Technology) and are shown in the Supplementary Table 1. PCR amplification of the *ssaA* gene was performed as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min. A final extension of 5 min at 72 °C was also done. The cloning procedure was performed by using the In-Fusion HD Cloning Kit (Clontech) according to the manufacturer's instructions. The pGEX-*ssaA* vector was used to transform *Escherichia coli* BL21 (DE3) cells. The production of heterologous protein was performed in *E. coli* cells cultured in LB medium until optical density of 0.4-0.45 at 660 nm was achieved by adding 0,5 M of Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1 h. The heterologous expression was evaluated in SDS-PAGE polyacrylamide gel stained with Coomassie blue (Sigma Aldrich).

2.10. Generation of polyclonal antibodies against SsaA protein

Polyclonal antibodies were obtained through inoculation of the fusion protein removed from SDS-PAGE in Balb/c mice, as previously described (Brito et al., 2011). A total of three immunizations was performed over an interval of 15 days. We used 100 µg of the SsaA fusion protein for each inoculation. The experiment was performed in triplicate, and pre-immune serum from each animal was obtained and stored at -20 °C in 50% glycerol. After the immunizations, the animals were euthanized in a CO_2 chamber and the sera containing polyclonal antibodies against SsaA (anti-SsaA) were obtained.

2.11. Western blot analysis

For Western blot experiments we used a total of 40 µg of the total protein extracts and of the exoproteomes from the isolates. The proteins were applied in SDS-PAGE and transferred to a nitrocellulose membrane.. The membrane was blocked with blocking solution (Phosphate buffer containing 5% (w/v) skimmed milk, 0.01% (v/v) Tween 20) for 2 h. The membrane was washed with a phosphate buffer and incubated with anti-SsaA at 1:1000 ratio, followed by incubation with anti-Mouse IgG coupled with alcaline phosphatase at 1:5000 ratio for 1 h. The reaction was revealed by *Fast* BCIP/NBT (5-bromo-4-cloro-3-indolylphosphate/nitroblue tetrazolium - Sigma-Aldrich), as previously described (Brito et al., 2011).

2.12. Amplification of ORF encoding SsaA from the S. saprophyticus isolates and DNA sequencing

The *ssaA* ORFs from the three *S. saprophyticus* isolates were amplified by PCR, as described above, and purified using the QIAquick PCR Purification (Qiagen). The PCR products were quantified by using

NanoDrop 2000 (ThermoScientific), and 300 ng of each PCR product was used for sequencing. DNA sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The samples were sequenced using a 3500 XL platform (*Thermo Fisher Scientific*). The reactions were performed in triplicate for each primer. The results were analyzed in the Phred/Phrad/Consed package with a phred20 quality threshold.

2.13. Real time PCR for quantification of SsaA transcripts (RT-PCRq)

The *S. saprophyticus* cells were cultured in the same condition used for proteomic analysis before RNA extraction. The RNA extractions were performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNAs were treated with DNAse I (Thermo Scientific, Foster City, CA, USA) prior the cDNA synthesis. The reverse transcription was performed using the RETROscript first strand synthesis kit (Thermo Scientific, Foster City, CA, USA) following the manufacturer's instructions. The cDNA target specificity of each primer pair was confirmed by melting curve analysis. The endogenous control of RT-PCRq reaction was performed using oligonucleotides that amplify a fragment of 30S ribosomal ORF sequence (accession number in NCBI database: gi|73661309, GeneID:3,615,961). The choice of the normalizing gene was evaluated by NormFinder Visual Basic application for Microsoft Excel (Andersen et al., 2004). The oligonucleotides used in this experiment are described in the Supplementary Table 1. The RT-PCRq reactions were performed in triplicate using a PowerUp SYBR Green Master Mix (Thermo Scientific, Foster City, CA, USA). Standard curves were generated by a 1:5 cDNA dilution. The standard curve method was used for relative gene quantification (Bookout et al., 2006).

3. Results

3.1. Evaluation of cell growth and viability

The cell growth was evaluated by monitoring optical density in spectrophotometry. The Supplementary Fig. 1 shows that *S. saprophyticus* cells remain in lag phase in the first 3 h and exponential phase occurs until around 9 h. After this point the cells remains in stationary phase until the 18 h analyzed. *S. saprophyticus* cells after incubation in BHI medium for 18 h were collected and incubated in fresh BHI medium and OD was monitored for 1, 3 and 6 h. The cell growth occurs in the first 3 h and after 6 h a reduction in OD occurs in the strain 9325, probably due to cell lysis (Supplementary Fig. 1). The time of 3 h was chosen for proteomic analysis. In order to check cell viability, these cells were evaluated by using flow cytometry. The *S. saprophyticus* cells were incubated in BHI medium for 18 h and then were transferred to a fresh BHI medium and cell viability was evaluated by flow cytometry after 3 h. Fig. 1 shows that culturing conditions used for proteomic analysis allow to obtain cells with high cell viability.

3.2. Overview of proteins secreted by S. saprophyticus

The exoproteome of the three isolates listed above were obtained. The protein samples were trypsin digested and peptides identified, as described above. A total of 119 proteins were identified in the ATCC 15,305 strain, 118 proteins in the 7108 strain, and 105 proteins in the 9325 strain. The protein abundance was evaluated analyzing the protein data from the experimental triplicate of the three strains. The dataset of the exoproteomes is available and shows the proteins identified by spectrometry analysis and the amount of protein quantified in ngram, as well as the quality analysis of the data (Oliveira et al., 2018).

Analysis of the three exoproteomes identified a total of 72 proteins secreted by all the three *S. saprophyticus* strains. Moreover, we identified proteins secreted by two isolates and other proteins secreted by only one of them. The number of proteins identified in each strain is shown in Fig. 2, Panel A. Fig. 2, Panel B shows a schematic representation of the proteins identified in the strains. The core of proteins identified in the analyzed strains includes proteins related to glycolysis and the pentose phosphate pathway (shown as moonlighting proteins), as well as proteins related to cell wall, iron capture and stress response. It is worth noting that the characterized Uro-adherence factor A (UroA) was not detected in the exoproteome of strain 9325 and the antigenic protein SsaA was not detected as a secreted protein in strain 7108. In addition, 7108 secretes proteases that were not detected in the other strains that were analyzed, and 9325 secretes proteins related to stress response that were also not detected in the other strains.

The fold change (in logarithm scale) of each protein's expression was calculated comparing its quantification in strains 7108 and 9325 with its level in the ATCC 15,305 strain. The list of proteins identified in the three *S. saprophyticus* isolates is laid out in Table 1. It was possible to detect 7 proteins related to glycolysis in the exoproteomes of the three *S. saprophyticus* isolates: enolase, glucose 6 phosphate isomerase, triosephosphate isomerase (TPI), glyceraldhyde 3 phosphate dehydrogenase (GAPDH), phosphoglycerate kinase and two fructose biphosphate aldolases (FBA), which were probably acting as moonlighting proteins in the exoproteome. Furthermore, we detected proteins related to the pentose phosphate pathway, such as transaldolase, transketolase and 6-phosphogluconate dehydrogenase decarboxilating protein.

Interestingly, we detected 7 proteins related to stress response in the exoproteome from the isolate analyzed, such as alkyl hydroperoxide reductase subunit C, catalase, thiol peroxidase, superoxide dismutase (SOD) [Mn/Fe] and thioredoxins. This demonstrates that *S. saprophyticus* presents a conserved machinery that is able to respond to stresses, including the response to oxidative damage in the host.

Three transglycosylases (IsaA and SceD1-2) were detected in the exoproteomes of the three *S. saprophyticus* strains. These proteins are related to cell wall synthesis and remodeling. IsaA is an immunodominant antigen related to methicillin resistance (Cordwell et al., 2002) and to the biofilm stabilization (Islam et al., 2015) in *S. aureus*.

It is important to mention that although we identified several proteins related to protein synthesis (33 ribosomal proteins and 7 other proteins) they do not seem to be related to cell lysis, since we monitored the cell viability (Fig. 1). Also, we calculated the percentage of proteins related to protein synthesis and the values were 4.9%, 7.8% and 5.7% in ATCC 15,305, 7108 and 9325, respectively (Oliveira et al., 2018). Secretion of ribosomal proteins are described in *S. aureus* and. These proteins do not possess signal peptides and they are probably secreted by a non-classical pathway (Sibbald et al., 2006).

The secretion of the classical virulence factor urease was detected in two strains in this proteomic analysis (ATCC 15,305 and 7108). It was not expected since all *S. saprophyticus* strains exhibit urease activity (Gatermann et al., 1989). However, since the *S. saprophyticus* cells were cultured in rich medium before our proteomic analysis, it explain the lower detection of this enzyme in the exoproteomes (0.25% of the ATCC 15,305 exoproteome and 0.77% of the 7108 exoproteome). In addition, the secretion of urease is stimulated by urea and our analysis were performed in BHI medium without this molecule and, for this reason, urease was not the focus of this work.

3.3. Most secreted proteins identified in S. saprophyticus strains

In order to identify the most abundant proteins secreted by *S. saprophyticus* strains, we evaluated the average amount (in nanograms) of each of them within the three exoproteomes. We obtained the percentages of the proteins that were secreted in the highest quantities, and the results are listed in Table 2. The 15 most plentiful proteins in the exoproteome of each strain corresponded to around 60–70% of the total of protein secretion. There were six proteins with the highest rates of

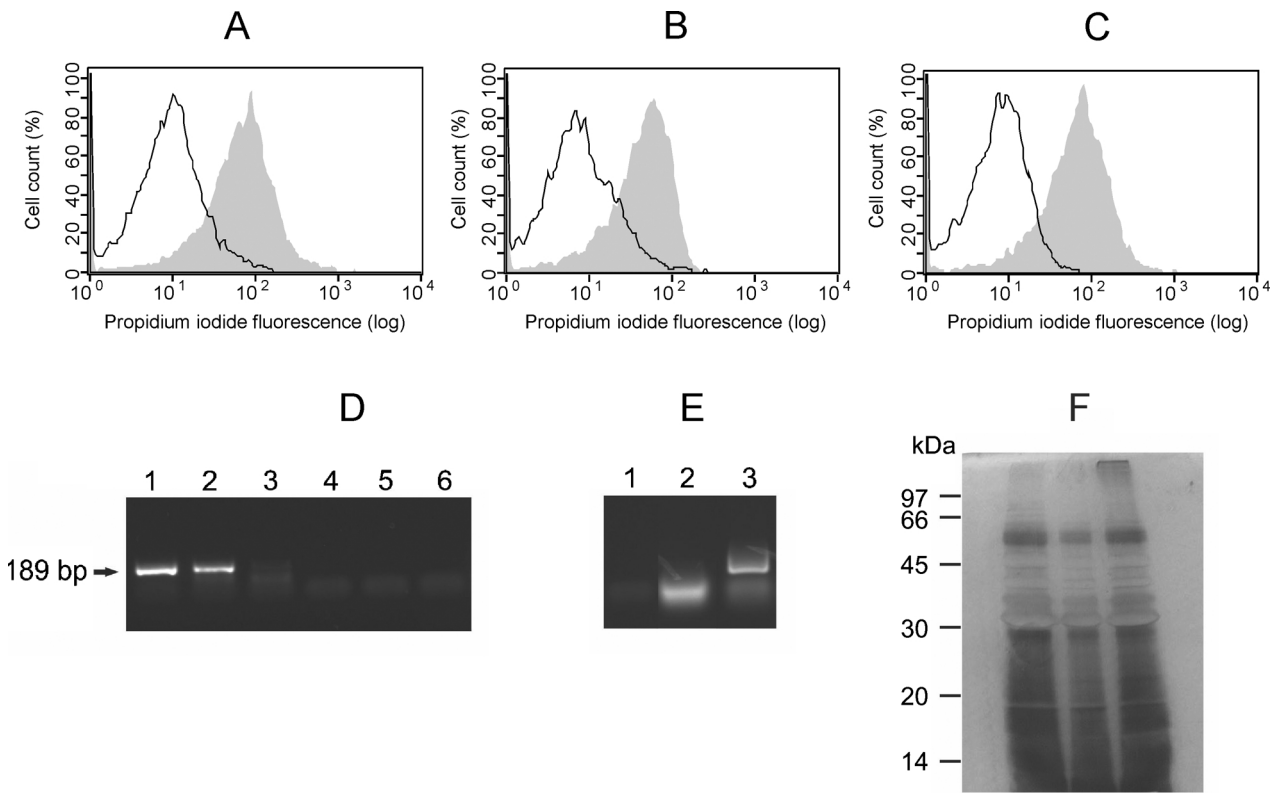

Fig. 1. Evaluation of *S. saprophyticus* cell viability, analysis of cell lysis and protein secretion profile of *S. saprophyticus*. The cell viability was evaluated to show the majority of cells used for proteomic analysis was not dead, using propidium iodide as dead cell marker, as described above. A: Cell viability of ATCC 15,305 cells. B: Cell viability of 7108 cells. C: Cell viability of 9325 cells. The grey peaks correspond to control dead cells and the clear peaks corresponds to the cells cultured in the same conditions used for proteomic analysis. D: PCR sensitivity for the *rpsa* gene was assessed using *S. saprophyticus* genomic DNA from ATCC strain 15,305 as a template (50 ng to 1 pg). PCR was performed with 5 concentrations of genomic DNA: 50 ng (lane 1), 5 ng (lane 2), 50 pg (lane 3), 5 pg (lane 4) and 1 pg (lane 5). A negative control without genomic DNA was also performed (lane 6). E: PCR performed with the culture supernatant (2 µL) of *S. saprophyticus* concentrated by a 3-kDa filter obtained after 1 h (lane 1), 3 h (Lane 2) and 6 h (lane 3) of incubation in BHI medium. F: Exoproteome profile of *S. saprophyticus* after 1 h (lane 1), 3 h (lane 2) and 6 h (lane 3).

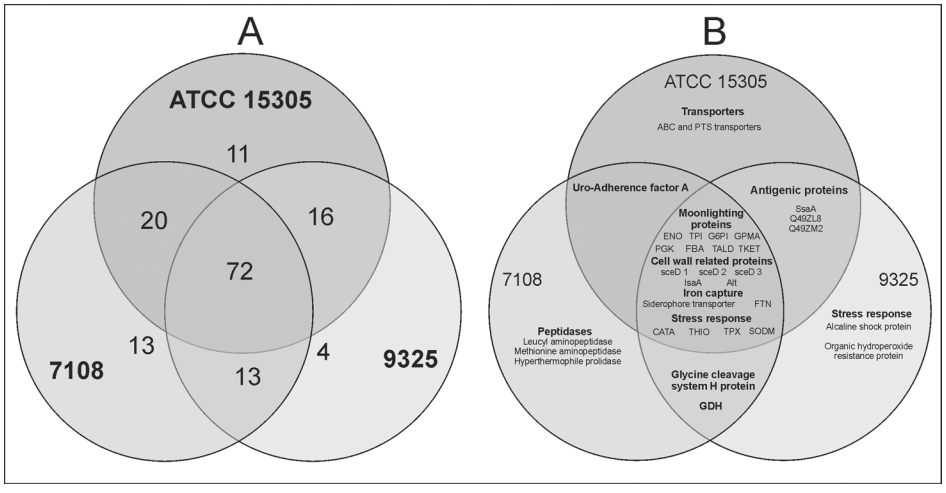

Fig. 2. Venn diagrams containing the number of proteins identified in the *S. saprophyticus* strains ATCC 15,305, 7108 and 9325 and schematic representation of the proteins identified in the exoproteomes. A: A total of 72 proteins were secreted by all the *S. saprophyticus* strains that were analyzed, 20 proteins were identified in strains ATCC 15,305 and 7108, 16 proteins were identified in strains ATCC 15,305 and 9325, and 13 proteins were identified in strains 7108 and 9325. A total of 28 proteins were identified in just one strain (13 proteins in 7108, 11 proteins in ATCC 15,305 and 4 proteins in 9325). B: ENO: enolase; TPI: triose phosphate dehydrogenase; G6PI: glucose 6 phosphate isomerase; TALD: transaldolases; TAKET: transketolases; GPMA: 2.3-bisphosphoglycerate-dependent phosphoglycerate mutase; PGK: phosphoglycerate kinase FBA: fructose biphosphate aldolases; sceD 1: transglycosylase sceD 1; sceD2: transglycosylase sceD 2; sceD3: transglycosylase sceD 3; IsaA: Immunogenic protein IsaA; ALT: autolysin; FTN: ferritin; CATA: catalase A; THIO: thioredoxin; TPX: thiol peroxidase; SODM: superoxide dismutase (Mn/Fe); GDH: glucose-1-dehydrogenase.

secretion in all of the analyzed strains: bifunctional autolysin, a 60 kDa chaperonin, transglycosylases IsA and sceD 2, and two uncharacterized proteins (accession numbers Q49VF9 and Q49VV4). Autolysin is the most secreted protein in the ATCC 15,305 and 9325 strains, corresponding to around 30% of the protein species secreted by them. Autolysin is also abundant in the exoproteome of strain 7108, corresponding to around 10% of all the protein species secreted by this isolate. In *S. aureus* the secretion of bifunctional autolysin is higher in virulent strains (Bonar et al., 2016). The role of autolysin in *S. saprophyticus* has been associated with cell wall remodeling during separation, and in *S. epidermidis* this protein is also described as acting as a binding protein (Heilmann et al., 1997; Hell et al., 1998).

The most abundant protein secreted by strain 7108 is uro-adherence factor A, corresponding to around 23% of the proteins secreted by this

Table 1
Proteins identified in all analyzed *S. saprophyticus* strains.

Accession number[1]	Protein description	Log FC[2]		t-statistic[3]		p-value[4]	
		7108	9325	7108	9325	7108	9325
Glycolysis							
ENO_STAS1	Enolase	0.64	0.54	3.07	2.59	0.03	0.09
G6PI_STAS1	Glucose-6-phosphate isomerase	−1.28	−0.24	−9.5	−1.77	≤ 0.01	0.25
TPIS_STAS1	Triosephosphate isomerase	0.37	−0.41	2.42	−2.67	0.07	0.09
Q49VZ9_STAS1	Glyceraldehyde-3-phosphate dehydrogenase	1.26	−0.69	8.69	−4.75	≤ 0.01	0.01
PGK_STAS1	Phosphoglycerate kinase	1.08	−0.14	5.72	−0.72	≤ 0.01	0.63
Q4A0Q6_STAS1	Fructose-bisphosphate aldolase class I	0.19	0.34	0.9	1.62	0.48	0.29
Q49Z72_STAS1	Fructose-bisphosphate aldolase class I	−0.12	0.34	−0.57	1.63	0.67	0.29
Piruvate dehydrogenase complex							
Q49WM1_STAS1	Dihydrolipoyl dehydrogenase	−2.02	−0.13	−7.39	−0.46	≤ 0.01	0.80
Lactic fermentation							
LDH_STAS1	L-lactate dehydrogenase	2.15	0.00	13.86	0.03	≤ 0.01	1.00
Pentose phosphate pathway							
Q49YL0_STAS1	Transaldolase	−0.98	−0.15	−8.27	−1.29	≤ 0.01	0.42
Q49XD6_STAS1	Transketolase	0.54	0.05	3.69	0.37	0.01	0.87
Q49XV6_STAS1	6-phosphogluconate dehydrogenase decarboxylating	−0.66	0.62	−5.16	4.83	≤ 0.01	0.01
C-compounds metabolism							
GPMA_STAS1	2.3-bisphosphoglycerate-dependent phosphoglycerate mutase	−1.70	−0.11	−9.59	−0.64	≤ 0.01	0.68
Q49ZH5_STAS1	Glucose-1-dehydrogenase	0.06	−0.84	0.13	−1.76	0.95	0.26
Fatty acid metabolism							
ACP_STAS1	Acyl carrier protein	−1.53	−0.05	−4.97	−0.16	≤ 0.01	1.00
Q49WB7_STAS1	3-oxoacyl-[acyl-carrier-protein] synthase	−1.38	−0.92	−4.29	−2.85	0.01	0.07
Isoprenoid metabolism							
ISPD_STAS1	2-c-methyl-d-erythritol 4-phosphate cytidylyltransferase	−1.66	−1.37	−4.35	−3.6	≤ 0.01	0.03
ATP synthesis							
ATPA_STAS1	ATP synthase subunit alpha	−0.81	−0.29	−5.68	−2.02	≤ 0.01	0.18
ATPB_STAS1	ATP synthase subunit beta	−1.46	0.43	−2.33	0.69	0.08	0.66
Nucleotide and nucleoside metabolism							
Q49Z85_STAS1	Purine nucleoside phosphorylase DeoD	−1.68	−0.19	−5.96	−0.66	≤ 0.01	0.66
Q49XS7_STAS1	Bacterial nucleoid DNA-binding protein	−1.66	−0.40	−14.07	−3.39	≤ 0.01	0.04
Amino acids metabolismo							
GCSH_STAS1	Glycine cleavage system H protein	−1.04	0.10	−4.4	0.42	0.01	0.83
Q49V22_STAS1	Cysteine synthase	−0.42	0.15	−0.88	0.32	0.49	0.90
Transcription							
Q49WH6_STAS1	Putative transcriptional regulator	1.58	2.09	3.76	4.98	0.01	0.01
Cell signaling							
Q49ZE7_STAS1	Adenylate kinase	−1.29	0.08	−4.21	0.26	0.01	0.94
Cell wall synthesis							
ISAA_STAS1	Probable transglycosylase IsaA	0.11	−0.36	0.23	−0.75	0.87	0.62
SCED1_STAS1	Probable transglycosylase sceD 1	1.40	−0.34	10.32	−2.5	≤ 0.01	0.10
SCED2_STAS1	Probable transglycosylase sceD 2	0.49	−0.97	1.52	−3	0.23	0.06
Q49WH3_STAS1	Bifunctional autolysin	1.35	0.00	14.55	0	≤ 0.01	1.00
Protein synthesis							
RS10_STAS1	30S ribosomal protein S10	1.25	0.05	2.58	0.1	0.06	1.00
RS16_STAS1	30S ribosomal protein S16	−0.79	0.32	−3.09	1.23	0.03	0.43
RL2_STAS1	50S ribosomal protein L2	−0.70	−1.06	−1.44	−2.2	0.25	0.15
RL6_STAS1	50S ribosomal protein L6	0.10	0.53	0.35	1.8	0.81	0.25
RL7_STAS1	50S ribosomal protein L7/L12	−1.30	0.16	−11.18	1.41	≤ 0.01	0.37
RL11_STAS1	50S ribosomal protein L11	−0.80	0.04	−5.26	0.27	≤ 0.01	0.93
RL15_STAS1	50S ribosomal protein L15	−0.58	0.38	−1.17	0.78	0.35	0.62
RL25_STAS1	50S ribosomal protein L25	−0.34	0.73	−1.87	4.07	0.15	0.02
RL29_STAS1	50S ribosomal protein L29	−0.98	−0.25	−3.81	−0.96	0.01	0.53
EFTS_STAS1	Elongation factor Ts	−0.55	0.65	−2.36	2.77	0.08	0.07
EFTU_STAS1	Elongation factor Tu	1.37	0.30	7.42	1.63	≤ 0.01	0.29
SYE_STAS1	Glutamate–tRNA ligase	−1.50	−1.32	−3.32	−2.91	0.02	0.06
RRF_STAS1	Ribosome-recycling factor	−1.22	0.15	−9.1	1.12	≤ 0.01	0.46
TIG_STAS1	Trigger fator	−0.51	0.12	−3.2	0.77	0.03	0.62
Protein folding. degradation and modification							
Q4A0H6_STAS1	Glutamyl endopeptidase	1.05	−0.64	7.65	−4.7	≤ 0.01	0.01
CLPP_STAS1	ATP-dependent Clp protease proteolytic subunit	−0.85	0.09	−8.23	0.9	≤ 0.01	0.55
Q49WC9_STAS1	Oligopeptidase F	−2.39	−1.09	−3.17	−1.45	0.03	0.36
CH10_STAS1	10 kDa chaperonin	−1.89	0.82	−7.07	3.06	≤ 0.01	0.06
CH60_STAS1	60 kDa chaperonin	0.48	1.27	2.72	7.12	0.05	≤ 0.01
GRPE_STAS1	Protein grpE	−1.12	0.84	−4.93	3.7	≤ 0.01	0.03
DNAK_STAS1	Chaperone protein DnaK	0.22	3.15	0.3	4.33	0.83	0.01

(*continued on next page*)

Table 1 (*continued*)

Accession number[1]	Protein description	Log FC[2]		t-statistic[3]		p-value[4]	
		7108	9325	7108	9325	7108	9325
Iron metabolismo							
FTN_STAS1	Ferritin	−1.45	0.52	−10.31	3.68	≤ 0.01	0.03
HEM3_STAS1	Porphobilinogen deaminase	0.00	0.34	0	1.13	1.00	0.46
HEMH_STAS1	Ferrochelatase	−0.64	−0.62	−4.8	−4.64	≤ 0.01	0.01
Transport							
Q49ZK8_STAS1	ABC-type cobalamin Fe3 + siderophore transport system	−0.80	−0.75	−2.78	−2.6	0.05	0.09
Stress response							
AHPC_STAS1	Alkyl hydroperoxide reductase subunit C	−1.88	−0.94	−15.42	−7.71	≤ 0.01	≤ 0.01
CATA_STAS1	Catalase	0.24	1.02	2.3	9.8	0.08	≤ 0.01
TPX_STAS1	Probable thiol peroxidase	−0.32	0.14	−1.59	0.68	0.21	0.66
Q49Z86_STAS1	Starvation-inducible DNA-binding protein	−0.03	0.63	−0.33	6.48	0.82	≤ 0.01
SODM_STAS1	Superoxide dismutase [Mn/Fe]	−1.51	0.09	−15.24	0.88	≤ 0.01	0.55
THIO_STAS1	Thioredoxin	−1.51	−0.28	−6.9	−1.3	≤ 0.01	0.42
Q49V79_STAS1	Beta lactamase	1.29	0.90	3.65	2.57	0.01	0.09
Virulence fator							
Q49ZM2_STAS1	Putative secretory antigen	0.99	−1.44	1.24	−1.81	0.33	0.25
Q4A0W2_STAS1	Immunodominant antigen	0.12	1.08	0.61	5.5	0.65	≤ 0.01
Uncharacterized proteins							
Q49ZY4_STAS1	Uncharacterized protein	−1.00	−1.10	−3.61	−3.98	0.02	0.02
Q49VC9_STAS1	Uncharacterized protein	0.62	−0.66	4.04	−4.26	0.01	0.01
Q49ZZ3_STAS1	Uncharacterized protein	0.48	−0.43	2.19	−1.94	0.10	0.21
Q49VV4_STAS1	Uncharacterized protein	−0.85	−0.21	−6.07	−1.49	≤ 0.01	0.34
Q49VF9_STAS1	Uncharacterized protein	0.19	−0.24	0.78	−0.98	0.55	0.53
Q49WI5_STAS1	Uncharacterized protein	0.41	0.55	0.72	0.95	0.58	0.53
Y954_STAS1	Uncharacterized protein	−1.45	−0.52	−11.75	−4.26	≤ 0.01	0.01
UP355_STAS1	Uncharacterized protein	−0.11	1.04	−0.34	3.34	0.81	0.04
Y2125_STAS1	Uncharacterized protein	−0.54	0.35	−2.12	1.38	0.11	0.38

[1] Accession number provided by Uniprot Database (http://www.uniprot.org/).
[2] Obtained from limma's topTable by subtracting the average expression in log2 scale against that of ATCC 15,305 strain.
[3] Estimate of the Student's t statistic comparing expression against that of the ATCC 15,305 strain.
[4] p-value from the Student's t distribution. Proteins with p-value ≤ 0.05 were considered regulated among the strains.

Table 2
Most secreted proteins secreted by *S. saprophyticus* strains (ATCC 15,305, 7108 and 9325).

Accession number	Protein description	ATCC 15,305 strain		7108 strain		9325 strain	
		ng	% in the exoproteome	ng	% in the exoproteome	ng	% in the exoproteome
Q49WH3_STAS1	Bifunctional autolysin	315.99	27.79	114.24	9.92	220.15	30.10
UAFA_STAS1	Uro-adherence factor A	114.91	10.11	259.04	22.49	–	–
LTAS_STAS1	Lipoteichoic acid synthase	45.50	4.00	–	–	17.77	2.43
CH60_STAS1	60 kDa chaperonin	45.33	3.99	30.29	2.63	15.89	2.17
ISAA_STAS1	Probable transglycosylase IsaA	41.50	3.65	29.66	2.58	42.22	5.77
SCED2_STAS1	Probable transglycosylase sceD 2	33.89	2.98	16.98	1.47	40.78	5.58
Q49UH9_STAS1	Mannosyl-glycoprotein endo-beta-N-acetylglucosamidase-like domain	32.49	2.86	–	–	–	–
Q49VF9_STAS1	Uncharacterized protein	30.95	2.72	23.10	2.01	26.16	3.58
Q49VV4_STAS1	Uncharacterized protein	26.88	2.36	34.85	3.03	22.67	3.10
Q49VC9_STAS1	Uncharacterized protein	24.65	2.17	*	*	27.10	3.71
DNAK_STAS1	Chaperone protein DnaK	21.10	1.86	14.27	1.24	–	–
LDH_STAS1	L-lactate dehydrogenase	19.02	1.67	*	*	15.24	2.08
Q49XD6_STAS1	Transketolase	18.19	1.60	*	*	13.31	1.82
Q49VZ9_STAS1	Glyceraldehyde-3-phosphate dehydrogenase	16.23	1.43	*	*	21.16	2.89
Q49XC4_STAS1	Uncharacterized protein	16.04	1.41	–	–	–	–
Q49WC9_STAS1	Oligopeptidase F	*	*	55.98	4.86	10.67	1.46
Q49WM1_STAS1	Dihydrolipoyl dehydrogenase	*	*	33.47	2.91	*	*
AHPC_STAS1	Alkyl hydroperoxide reductase subunit C	*	*	18.93	1.64	*	*
Q49ZZ3_STAS1	Uncharacterized protein	*	*	*	*	14.10	1.93
EFTS_STAS1	Elongation factor Ts	*	*	16.56	1.44	*	*
Q49YL0_STAS1	Transaldolase	*	*	15.35	1.33	*	*
SCED1_STAS1	Probable transglycosylase sceD 1	*	*	*	*	12.79	1.75
Q49VK7_STAS1	Secretory antigen SsaA-like protein	*	*	–	–	10.78	1.47
DNAK_STAS1	Chaperone protein DnaK	*	*	14.27	1.24	–	–
SODM_STAS1	Superoxide dismutase [Mn/Fe]	*	*	14.17	1.23	*	*
Total		802.67	70.59	691.16	60.01	510.78	69.85

*Detected but not included in the list of the 15 most abundant proteins in this strain.
Not detected in this strain.

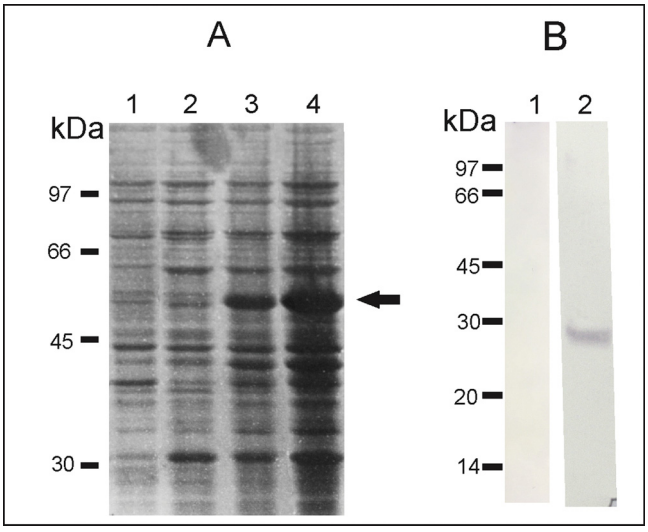

Fig. 3. Heterologous expression of SsaA protein from *S. saprophyticus* and polyclonal antibodies obtainment. A: Heterologous expression of SsaA fused to GST protein in the *E. coli* system. The protein extracts were obtained without IPTG (lane 1), and then again later, after the addition of IPTG 0.5 mM (lanes 2–4). Extracts were taken after 30 min (lane 2), 1 h (lane 3) and 2 h (lane 4) of incubation with IPTG. The black arrow indicates the band corresponding to recombinant SsaA fused to GST protein used for polyclonal antibodies obtainment. B: Western blot to test specificity of polyclonal antibodies. Anti-SsaA were obtained after inoculation of recombinant protein in mice, as described. Negative control performed with pre-immune sera was performed (lanes 1). The specificity of the polyclonal antibodies was shown performing Western blot with protein extract from *S. saprophyticus* ATCC 15,305 strain, showing reaction with one protein specie with molecular weight compatible with the predicted molecular size of SsaA (lane 2).

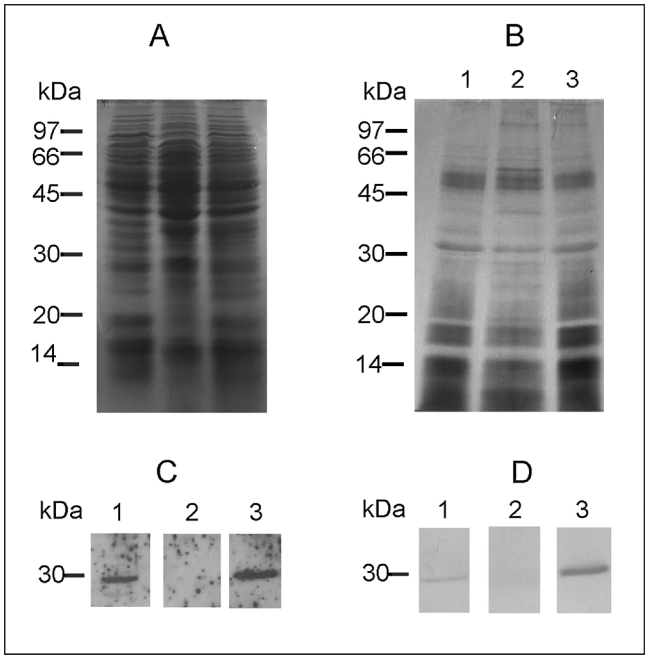

Fig. 4. Western blot using anti-SsaA with exoproteome of *S. saprophyticus* strains ATCC 15,305, 7108 and 9325. A: Total protein extract from *S. saprophyticus* strains ATCC 15,305 (lane 1), 7108 (lane 2) and 9325 (lane 3). B: Protein extract from culture supernatant obtained from the *S. saprophyticus* strains ATCC 15,305 (lane 1), 7108 (lane 2) and 9325 (lane 3). **C:** Western blot made by using anti-SsaA in total protein extract from *S. saprophyticus* strains ATCC 15,305 (lane 1), 7108 (lane 2) and 9325 (lane 3). D: Western blot culture supernatant obtained from the *S. saprophyticus* strains ATCC 15,305 (lane 1), 7108 (lane 2) and 9325 (lane 3).

isolate. Uro-adherence factor A is also secreted by strain ATCC 15,305, being the second most secreted protein in this strain (10.11% of the exoproteome). However, uro-adherence factor A was not detected in the exoproteome of strain 9325. Among all the proteins secreted in abundance by each strain (15 proteins of each isolate), three were only secreted by two isolates: uro-adherence factor A (not secreted by the 9325 strain), lipoteichoic acid synthase (not secreted in the 7108 strain) and a chaperone DNA K protein (not secreted by the 9325 strain). These results show that the secreted proteins vary in abundance, and, in some cases, proteins are not secreted by all the isolates.

3.4. Validation of proteomic results – western blot to evaluate SsaA secretion

In order to validate the proteomic results, we selected the SsaA protein, which was detected in the exoproteome of two isolates (ATCC 15,305 and 9325) and was not detected in the exoproteome of the 7108 strain. The gene encoding the SsaA protein was obtained in GeneBank, cloned and then the protein was expressed in a heterologous system. The expressed protein was used to inoculate Balb/C mice to obtain polyclonal antibodies (anti-SsaA). Fig. 3 shows the heterologous expression and the obtainment of anti-SsaA polyclonal antibodies. The production of anti-SsaA polyclonal antibodies was performed in triplicate. Three animals were used to obtain polyclonal antibodies anti-SsaA and the sera were tested independently in three concentrations: 1:500 (lanes 2), 1:750 (lanes 3) and 1:1000 (lanes 4), showing similar results (Supplementary Fig. 3).

Afterwards, we used the anti-SsaA antibodies to evaluate the production and secretion of SsaA by the *S. saprophyticus* strains. Fig. 4 shows that SsaA is detected in strains ATCC 15,305 and 9325, both in the cytoplasm (Panel C) and in the exoproteome (Panel D), but not in the 7108 strain (neither in the cytoplasm nor in the exoproteome). These results corroborate the proteomic data. In addition, as evaluated by the proteomic approach, strain 9325 was found to secrete higher levels of SsaA compared with ATCC 15,305.

In order to evaluate if strain 7108 possess the ORF encoding SsaA protein, we performed a PCR reaction to amplify this ORF. The amplified fragment was sequenced and the results show that all *S. saprophyticus* strains analyzed (ATCC 15,305, 7108 and 9325) present amplification of the ORF encoding SsaA protein. The amplicons were sequenced and no alteration in the nucleotide sequences were detected among the strains (Supplementary Fig. 2). The NCBI database also contains a previous draft from the 7108 genome and the gene encoding SsaA is sequenced without mutation in this data (accession number NZ_LMYQ00000000, Locus tag ASS79_RS03200). These data suggest that the inability of the 7108 strain to produce and secrete SsaA protein is not related to a coding sequence alteration, but is more likely related to transcriptional and/or post-transcriptional events.

Since the gene encoding SsaA was encountered in the three *S. saprophyticus* strains, we performed RT-PCRq experiment to evaluate the production of transcripts encoding SsaA protein in the strains analyzed. The result is shown in the Fig. 5. The *S. saprophyticus* ATC15305 produces lower amounts of the transcript encoding SsaA protein. On the other hand, the strain 9325 produces higher amounts of the transcripts (30-fold compared to ATCC 15,305). This result corroborates proteomic analysis suggesting that the high secretion of SsaA in the strain 9325 is a function of increased mRNA production or higher stability of this molecule in this strain. The *S. saprophyticus* 7108 strain also produces transcripts encoding SsaA at high level (20-fold compared to ATCC 15,305 strain). The result suggests post-transcriptional events regulates the production and secretion of SsaA protein in the 7108 strain.

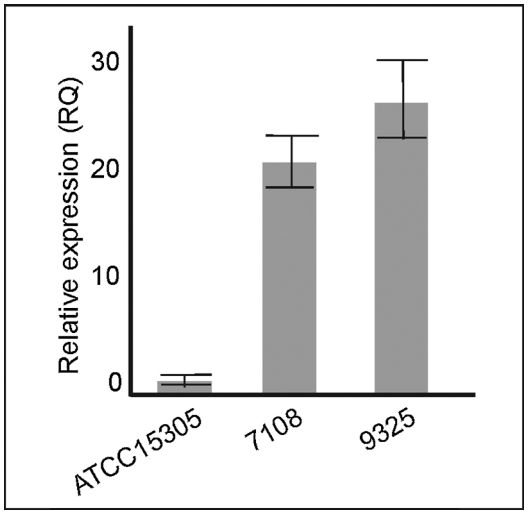

Fig. 5. Quantitative real time PCR (RT-PCRq) to quantify transcripts encoding SsaA in the *S. saprophyticus* strains. The RT-PCRq reactions were performed with cDNAs from three *S. saprophyticus* strains. The values were normalized using transcripts encoding 30S ribosomal protein. The reactions were performed in triplicate and standard deviation are shown.

3.5. Abundance of some proteins in the S. saprophyticus exoproteomes

We detected that strain 7108 secreted higher amounts of proteases when compared with the other strains. In order to quantify the percentage of proteases secreted by each *S. saprophyticus* strain, we calculated the percentage of proteases secreted by all three strains (identified in at least two replicates), as demonstrated in Fig. 6, Panel A. The percentage values were obtained from the amount of proteins (in ngram) identified in each strain. From the total number of proteins secreted by ATCC 15,305, 1.25% are proteins classified as proteases and peptidases; in strain 9325 we identified that 2.35% of the exoproteome is composed of proteases and peptidases. On the other hand, strain 7108 secreted a higher content of proteins identified as proteases and peptidases, with a percentage of 7.24.

In the same way, we also evaluated the percentage of proteins that were classified as antigenic. These proteins were detected in higher amounts in strain 9325, as shown in Fig. 6, Panel B. From the total number of proteins secreted by strain 9325, 4.46% correspond to four proteins described as antigenic (an immunodominant antigen Q4A0W2, two secretory antigens Q49ZM2 and Q49ZL8 and the staphylococcal secretory antigen SsaA). These same proteins were identified in strain ATCC 15,305, but only amounted to 1.89% of the total number of proteins secreted by this strain. The amount of antigenic proteins secreted by strain 7108 is lower (0.26%), and this strain does not secrete the SsaA protein in a detectable level, as is demonstrated by the Western blot analysis (Fig. 4). The secretion of the antigenic proteins Q49ZM2 and Q49ZL8 was also not detected by proteomic approach in strain 7108.

The secretion of transglycosylases was also evaluated by comparing the three *S. saprophyticus* strains. Three transglycosylases were detected in the three isolates (IsaA and sceD1-2). However, IsaA, sceD 1 and sceD2 were secreted in higher amounts in strain 9325. The analysis of percentage of secretion is shown in Fig. 6, Panel C, and reveals that from the total number of proteins secreted by the 9325 strain, 13.1% corresponds to transglycosylases. In strains ATCC 15,305 and 7108 the percentage of secretion of transglycosylases is 7.68 and 4.36, respectively. The transglycosylase secretion content probably reflects the polysaccharide content outside the cell, since strain 9325 possess the highest polysaccharide capsule, followed by ATCC 15,305 and 7108, respectively. It is important to point out that the presence of capsules in different sizes is reported in *S. saprophyticus* strains, and that more than one capsular serotype exists (Park et al., 2010).

4. Discussion

The heterogeneity of *S. saprophyticus* strains has been described in terms of genotypic and phenotypic variations among strains isolated from humans and animals. Analysis of animal and human *S. saprophyticus* strains reveals that strains from both animal and clinical sources presents classical virulence and adherence factors. However, the ability to adhere to collagen I was higher in clinical strains (Kleine et al., 2010). The genotypic variation among coagulase negative *Staphylococcus* species strains isolated from inpatients and outpatients was also reported by using pulsed-field gel electrophoresis typing (Talebi et al., 2016).

The presence of capsule in *S. saprophyticus* strains have been reported and it is attributed to the capsule gene cluster cap_{Ssp}. However, non-capsular clinical strains are also reported. Since clinical non-capsular strains have been reported, the capsule is not essential for infection in human host and it is presented only in 1.3% of clinical *S. saprophyticus* strains, suggesting other aspects can be involved during pathogenesis. In order to enlarge the characterization of *S. saprophyticus* strains, our study presents, for the first time, large-scale identification of proteins secreted by three *S. saprophyticus* strains. In addition, we compared the exoproteome profiles of the three strains and detected diversity in them. Our study identified proteins secreted in the

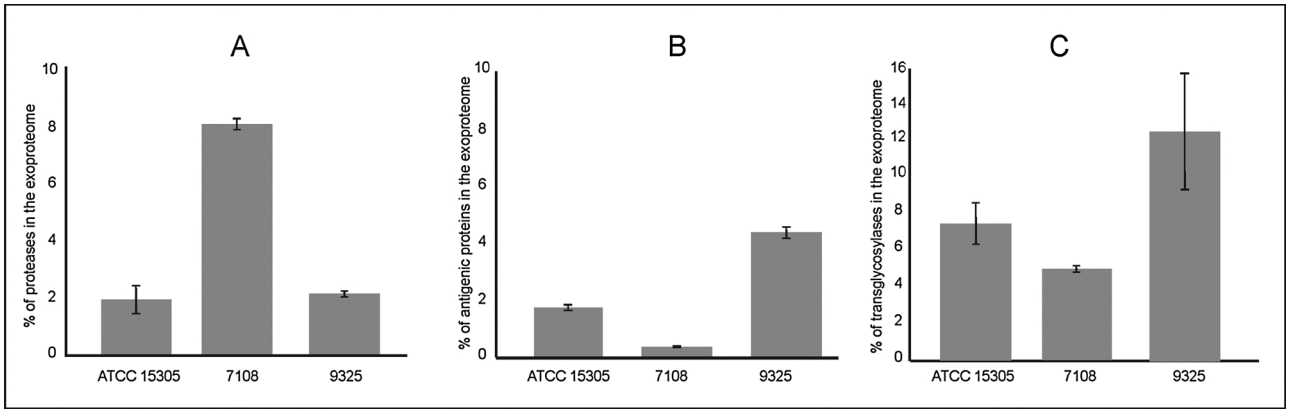

Fig. 6. Abundance of proteases, classical antigenic proteins and transglycosylases in the exoproteomes from *S. saprophyticus* strains ATCC 15,305, 7108 and 9325. The total number of proteins identified (in nanogram) was calculated, and the amount of proteins corresponding to proteases, antigenic proteins and transglycosylases was obtained. The error bars corresponds to standard error of mean. A: Percentage of proteins identified as proteases in the exoproteomes analyzed. B: Percentage of proteins described and antigenic proteins in the exoproteomes analyzed. C: Percentage of transglycosylases in the exoproteomes analyzed.

stationary phase of growth, and proteins related to several biological functions were detected.

A core of secreted proteins was detected. These proteins have been found in various studies on the *S. aureus* exoproteome (Burlak et al., 2007; Muthukrishnan et al., 2011). These proteins can play different roles extracellularly as moonlighting proteins and includes glycolytic enzymes (GAPDH, TPI, ENO, FBA and phosphoglycerate kinase), proteins related to pyruvate dehydrogenase complex and other enzymes related to energy metabolism, such as glucose 1 dehydrogenase, from Entner-Doudoroff pathway. For example, enolase is related to plasminogen binding in several streptococcal species such as *Streptococcus oralis* (Kinnby et al., 2008). GAPDH is also encountered on the cell surface and outside the cell in several bacterial species, including *Streptococcus pneumonie* and *Lactobacillus crispatus* (Bergmann et al., 2004; Antikainen et al., 2007). Recently, studies comparing *S. aureus* community-associated (CA) and hospital-associated (HA) strains identified that HA strains secretes moonlighting proteins at higher levels when compared to CA strains, suggesting the secretion of moonlighting proteins increases the virulence and survival ability (Mekonnen et al., 2017).

Other proteins were detected in two or one of the strains, suggesting heterogeneity in the content of the exoproteome occur among the strains (Fig. 2, Panel A). Differences in proteomic profiles in *S. epidermidis* were also detected in cells cultured during biofilm formation and in glucose-enriched medium (Carvalhais et al., 2015). Comparative analysis using several *S. aureus* strains depicted a core of secreted proteins and several proteins are secreted only by one or a few isolates, showing that heterogeneity exists among isolates (Sibbald et al., 2006).

Other virulence factor detected in our analysis is the UafA protein, associated to adherence event in the host. This protein was not detected in the exoproteome from 9325 strain, but is included in the list of the most secreted protein in the strains ATCC 15,305 and 7108 (Table 2). Previous work shown that the strains ATCC 15,305, 7108 and 9325 possess the gene *uafA* (Kleine et al., 2010). We speculate that the capsule can inhibit the secretion of UafA and it is supported by the fact that the adherence mediated by UafA is inhibited by capsule (Kuroda et al., 2005). Special note must be made of the fact that we identified that the antigenic protein SsaA was secreted by the isolated strains ATCC 15,305 and 9325, but not by 7108. This data was confirmed by Western blot analysis (Fig. 4), and we also evaluated the presence of the ORF encoding SsaA protein without mutation in the three strains (Supplementary Fig. 2). Surprisingly 7108 produces transcripts encoding SsaA at high level (Fig. 5) suggesting the absence of production of the SsaA protein occurs due post-transcriptional events. The post-transcriptional regulation occurs in bacteria and can be regulated by small RNAs (sRNA). *Pseudomonas aeruginosa* possess post-transcriptional regulation mediated by one sRNA named RgsA and requires the RNA chaperone Hfq to regulate some target, such as the alternative sigma factor RposS (Lu et al., 2018). The function of SsaA is not completely clear. Transcripts encoding SsaA protein are overexpressed in *S. aureus* and *S. epidermidis* during infection in *Caenorhabditis elegans* (JebaMercy et al., 2015). Furthermore, the secretion of SsaA protein is higher in planktonic *S. aureus* cells in comparison with cells recovered from biofilm (Resch et al., 2006). In *S. epidermidis*, higher amounts of anti-SsaA IgG are present in patients' sera during endocarditis, but not with other *S. epidermidis* infections (Lang et al., 2000). The evaluation of SsaA secretion by other *S. saprophyticus* strains, and the comparison with data obtained from experimental infection in animal models, are necessary to contribute to the elucidation of the role of this protein during the infective process in *S. saprophyticus*. The elucidation of post-transcriptional regulation in *S. saprophyticus* is also needed and can contribute to understand the metabolic flexibility in this model.

In order to evaluate the major differences occurring in the exoproteome profile we screened the most regulated proteins among the three strains. We detected that they differs in the secretion of proteases, antigenic proteins and transglycosylases (Fig. 6). The strain 7108 secretes higher content of proteases compared to the other two strains. Proteases are specially important during infection and secreted proteases from *S. aureus* are associated with degradation of host immune system proteins (Stapels et al., 2017) and can be used during degradation of epithelial cells (Murphy et al., 2018). The strain 9325 secretes higher level of antigenic proteins and transglycosylases (followed by ATC 15,305 and 7108). The function of the antigenic proteins is not completely known but the absence of these antigenic proteins can act as an immune system evader while the presence of these antigenic proteins can enhance phagocytosis by the host (that is especially important for intracellular pathogens). Proteomic analysis comparing two *Mycobacterium tuberculosis* strains also detected differences in the secretion of antigenic protein. These proteins may play a role in evasion of host immune responses (antigenic variation) and the ability of secretion of antigenic protein can reflect that different strains can use different mechanism to interact with host immune system (Cornejo-Granados et al., 2017). In addition, strain 9325 secretes higher level of transglycosylases. These proteins besides act in the cell wall synthesis, and are crucial in the complement activation mediated by IgG in *S. aureus* (Lee et al., 2015). Together, these results show *S. saprophyticus* present variation in the relative abundance of proteins related to host-interaction process and suggest that strains can use more than one strategy during interaction with human cells.

Heterogeneity in the exoproteome content described in this study for *S. saprophyticus* uropatogenic strains is also reported in other species such as *S. aureus* and *S. epidermidis*, and is associated with genomic plasticity and variant gene regulation (Ziebandt et al., 2010). Proteomic studies, using two-dimensional gel electrophoresis (2-DGE) with *S. aureus* strains isolated from different sites of infection, identified 12 proteins detected only in isolates from bacteremia, suggesting that the ability to secrete some proteins can be related to the ability to cause infection in the host (Liew et al., 2015). The comparative exoproteome was also performed using three *S. epidermidis* and several proteins predicted to be secreted by classical and non-classical pathways were detected. In addition, several strain-specific proteins were detected (Siljamaki et al., 2014).

This study presents for the first time the proteomic approach to investigate the exoproteome profile of *S. saprophyticus*. Furthermore, we validate the proteomic data by using anti-SsaA polyclonal antibodies. Differences detected among the proteins secreted by theses strains were discussed and these data will contribute towards enlarging the knowledge of *S. saprophyticus* biology, as well as in the identification of strain-specific factors that are able to contribute to virulence and adaptation. Further analysis is required in order to elucidate if the secretion process used by these *S. saprophyticus* strains can explain differences in the exoproteome profiles and whether secretion can be related to *S. saprophyticus*'s ability to cause infection in its host.

5. Conclusions

The *S. saprophyticus* strains analyzed in this work presented a core of secreted proteins, common in the strains. Otherwise, some proteins related with adhesion (for example, Uro-adherence factor A), and virulence factors, such as proteases and antigenic proteins, are not secreted by all the *S. saprophyticus* strains analyzed, demonstrating that these strains possess versatility in the exoproteome profile.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank Juliano Domiraci Paccez for his helpful suggestions. We thank Alex Jesus de Carvalho (*in memorian*) for his helpful contribution and kind relationship in the research group. This work was supported

by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process number 444662/2014-6) and Fundação de Amparo á Pesquisa do Estado de Goiás (FAPEG, Pronex). ASO, IIRR and LSO were supported by scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.08.008.

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