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The potential role of Staphylococcal enterotoxin C and TSST-1 in the infection of bovine mammary epithelial cells

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ABSTRACT

Bovine mastitis is a continuous problem in the dairy industry. The infection has significant impact on health and welfare of animals and causes financial losses for the farmers and the dairy industry. A key bacterium associated with bovine mastitis is *Staphylococcus aureus (S. aureus)*. It produces different exotoxins including more than 24 different staphylococcal enterotoxins and toxic shock syndrome toxin-1 (TSST-1). We have investigated the role of enterotoxin C (SEC) and TSST-1 in the infection process. Five different *S. aureus sec* or *tst-1* deletion mutants, derived from three wild type strains isolated from cases of bovine mastitis, were constructed and characterized regarding growth and enterotoxin formation. These mutant and wild type strains were used to infect bovine mammary epithelial (BME-UV) cells to evaluate their infection ability. The ratio of *S. aureus* recovered in BME-UV cell lysate after 7 h of infection to the initial *S. aureus* infection dose was calculated to provide a measure of infection capability of each strain. Deletion of the *sec* gene overall showed a reduction in infection ratio, suggesting that presence of SEC may play a role during the establishment of infection. In contrast, deletion of *tst-1* did not appear to affect the infection capability to the same extent. Proteomic analyses indicated that infection by two out of three *S. aureus* wild type strains elicited a systematic alteration in the BME-UV cell proteome. The isogenic *sec* deletion mutants of these two wild type strain also produced differences in the proteome of the BME-UV cells compared to the wild-type infected cells. Altogether the reduced infection ratios and altered protein profiles suggest that SEC can play a role in the *S. aureus* infection process of BME-UV cells while a role for TSST-1 still remains unclear. Further investigations of their specific functions is important to elucidate if these toxins are potential targets in new preventive strategies or treatments for bovine mastitis.

1. Introduction

One of the most common and costly problems in the dairy industry is bovine mastitis. Mastitis is an inflammatory reaction in the bovine mammary tissue, most often in response to bacterial invasion. The infection can manifest itself as either a clinical or a subclinical form [1]. Especially acute clinical mastitis is commonly treated with antibiotics [2]. Bovine mastitis not only affects individual animals, but also places the health of the whole herd at risk.

Staphylococcus aureus is one of the most common pathogens causing contagious mastitis [3]. *S. aureus* produces different virulence factors and exotoxins including enterotoxins and toxic shock syndrome toxin-1 (TSST-1). Currently, more than 24 different staphylococcal enterotoxins (SEs) and enterotoxin-like proteins (SEls) have been identified and characterized [4–9]. Both enterotoxins and TSST-1 are known as pyrogenic toxin superantigens, which are recognized to have potent effects on the immune response of the host organism, and play an important role in establishing and maintaining infections [10,11].

The enterotoxin C and TSST-1 genes (*sec* and *tst-1* respectively) are the toxin genes most frequently encountered in the *S. aureus* strains isolated from dairy cows [12–18]. Both genes are often located in the same type of mobile genetic element, the staphylococcal pathogenicity

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island (SaPI) [8]. The genes encoding SEC and TSST-1 have been identified in 20 % of bovine-associated *S. aureus* strains [19]. Both genes are located 2.2 kb apart in the same SaPI known as $SaPI_{bov}$ [20].

An association between *S. aureus* isolated from peracute or severe bovine mastitis and the production of TSST-1 and SEC was early demonstrated [21,22]. In a study from 2019, Fang et al. showed that the injection of SEC into mouse mammary glands resulted in a severe inflammation, which could be inhibited by anti-SEC antibodies [23]. In addition, several earlier studies have been published on the effect of vaccination using inactivated SEC and TSST-1 on the extent of *S. aureus* bovine mastitis and nasal colonization as well as systemic infection in a mouse model [24–26]. These findings overall indicate a role of SEC and TSST-1 in mastitis caused by *S. aureus*.

The aim of the current study was to further investigate the contribution of SEC and TSST-1 to the infection process of bovine mammary cells. Three *S. aureus* strains, belonging to commonly demonstrated sequence types (STs) associated with acute clinical bovine mastitis in Sweden were used [27]. To evaluate the role of the toxins on infection ability, *sec* and *tst-1* deletion mutants were constructed and infection studies were performed with the wild type and isogenic mutant *S. aureus* strains in a model based on a bovine mammary epithelial cell line (BME-UV). In addition, protein expression profiles in infected BME-UV cells were analyzed using a proteomic approach to further evaluate the role of the two toxins.

2. Materials and methods

2.1. Bacterial strains and initial wild type gene sequence characterization

Three different *S. aureus* wild type strains were used in this study: MAS106, MAS602 and MAS660, which all contained both *sec* and *tst-1* genes [28]. The strains belong to the sequence types (STs): ST 504, ST 3140 and ST3140, respectively (Table 1). These STs all belong to CC151, also denoted CC705, and are among the most common sequence types of *S. aureus* isolated from acute clinical bovine mastitis in Sweden [27]. Before constructing deletion mutants of *sec* and *tst-1*, the DNA sequence of *sec* and *tst-1* genomic regions from MAS106, MAS602 and MAS660 were investigated. Primers to sequence *sec* and *tst-1* regions were designed based on the DNA sequence alignment of five known *S. aureus* strains (ED133, Mu3, N315, Mu50, MW2) with multiple sequence comparison by log-expectation (MUSCLE) [29] (Table 2).

Table 1
Bacterial strains and plasmids used in this study. ST = Sequence type.

Bacterial strain/ plasmids	Characteristics	Source
S. aureus		
MAS106	ST 504	Swedish Veterinary Agency, Sweden
MAS602	ST 3140	Swedish Veterinary Agency, Sweden
MAS660	ST 3140	Swedish Veterinary Agency, Sweden
MAS106Δ*sec*	MAS106 with Sp^r replacing *sec*	This study
MAS106Δ*tst-1*	MAS106 with Sp^r replacing *tst-1*	This study
MAS602Δ*sec*	MAS602 with Sp^r replacing *sec*	This study
MAS602Δ*tst-1*	MAS602 with Sp^r replacing *tst-1*	This study
MAS660Δ*sec*	MAS660 with Sp^r replacing *sec*	This study
Escherichia coli		
DC10B	*dcm* mutant of high-efficiency cloning strain of *E. coli* DH10B	[31]
Plasmids		
pDG1727	pMTL23 derivative; Ap^r Sp^r	[30]
pIMAY	pIMC5 with tetracycline; inducible secY antisense from pKOR1; Cm^r	[31]
pIMAY Δ*sec*	pIMAY with the construct to replace *sec* by Sp^r	This study
pIMAY Δ*tst-1*	*pIMAY* with the construct to replace *tst*-1 by Sp^r	This study

Table 2
Primers used for sequencing the *sec* and *tst-1* genomic regions.

Target DNA	Primer name and orientation	Primer sequence 5′ to 3′
0.4 kb upstream of *sec*	YB-0070 (F)	CTTCGGTACTTGCCTATTATTATA
0.2 kb from the start of *sec*	YB-0071 (R)	CAGACTTAACTTTAGTTGCTGATACA
0.2 kb from the start of *sec*	YB-0072 (F)	GATGAAGTAGTTGATGTGTATGG
0.2 kb from the end of *sec*	YB-0073 (F)	GGTATGATATGATGCCTGC
0.7 kb downstream of *sec*	YB-0074 (R)	GAATATATAACTAGTGATCTAAAGGG
0.7 kb upstream of *tst-1*	YB-0075 (F)	TTTCATTTGTGATTGNAGTTG
0.1 kb from the start of *tst-1*	YB-0076 (R)	ATTAGATGATAAGGGAACAGG
0.1 kb from the start of *tst-1*	YB-0077 (F)	CCTGTTCCCTTATCATCTAAT
0.15 kb from the end of *tst-1*	YB-0078 (F)	GGTGGTTATTGGAAAATAACA
0.3 kb downstream of *tst-1*	YB-0079 (R)	TAACGGNAATGTGCCTATAT

Prior to DNA extraction, the wild type strains were grown overnight in brain heart infusion (BHI) broth (Difco Laboratories, BD Diagnostic Systems, Le Point de Claix, France) at 37 °C and 200 rpm. Their chromosomal DNA was extracted using Chelex 100 molecular biology grade resin (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendation.

In order to amplify *sec* and *tst-1* regions for sequencing purpose, PCR was performed on a Gene Amp 9700 thermal cycler (PerkinElmer Cetus; Norwalk, CT, USA). The reaction contained genomic DNA from each wild type strain as a template, primer set YB-0070-YB-0074 for *sec* region and YB-0075-YB-0079 for *tst-1* region, and proofreading enzyme Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 1 × Phusion GC Buffer. The concentrations of the PCR components were as recommended by the manufacturer. The cycling steps included initial denaturation at 98 °C for 3 min, followed by 30 cycles of denaturation at 98 °C for 20 s, annealing at 52.5 °C for 30 s, and elongation at 72 °C for 100 s, and a final extension step at 72 °C for 7 min.

PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). The purified DNA was diluted to 10 ng × μl^{-1}, premixed with 2 μl of a 10 μM solution of the appropriate primer, and sequenced by Eurofins Genomics, Ebersberg, Germany. By overlapping regions, the individual sequence reads were assembled, reviewed and confirmed manually using Serial Cloner 2.6.1 (Franck Perez, Serial Basics) and FinchTV 1.5.0 (Geospiza Inc., PerkinElmer, Norwalk, CT, USA).

2.2. Construction of the sec and tst-1 deletion mutants

To construct the Δ*sec* and Δ*tst-1* deletion mutants, primers were designed with an addition of a specific restriction site in their 5′ end (*Sac*I, *Not*I, *Eco*RI or *Sal*I) (Table 3). These primers were used to amplify approximately 500–650 bp fragments of homologous sequences upstream and downstream of the *sec* and *tst-1* genes. PCR to amplify upstream and downstream homologous sequences of the *sec* and *tst-1* was performed using genomic DNA from each wild type strain as a template, and Phusion DNA polymerase at a final concentration of 1 × Phusion GC Buffer. PCR cycling steps included initial denaturation at 98 °C for 3 min, 15 cycles of denaturation at 98 °C for 20 s, annealing at 50 °C for 30 s and elongation at 72 °C for 1 min, 20 cycles of denaturation at 98 °C for 20 s, annealing and elongation at 72 °C for 1 min, and final extension at 72 °C for 7 min.

The spectinomycin resistance cassette (Sp^r) was used as a

Table 3
Primers used in this study for Δ*sec* and Δ*tst-1* deletion mutant construction.

Function	Primer name	Primer sequence 5′ to 3′	Reference and restriction site
Amplification of homology fragments for pIMAY Δ*sec*	YB-0084 (F)	AGTCAGTCGAGCTC TAAGGCATTTAATATTGAGTTG	This study, *Sac*I
	YB-0085 (R)	AGTCAGTCGCGGCCGC GCTAATACGTTGGGTGTAAA	This study, *Not*I
	YB-0086 (F)	AGTCAGTCGAATTC AAGGTAAGTACTTCGGTGCT	This study, *Eco*RI
	YB-0087 (R)	AGTCAGTCGGTCGAC TCTCGTACTATATATGGCGG	This study, *Sal*I
Amplification of homology fragments for pIMAY Δ*tst-1*	YB-0080 (F)	AGTCAGTCGAGCTC TTCATCGCGTTGTTTAAGT	This study, *Sac*I
	YB-0081 (R)	AGTCAGTCGCGGCCGC TAAATGAATCAATTAACCATCTCT	This study, *Not*I
	YB-0082 (F)	AGTCAGTCGAATTC TTTCCAAATAAGTGGTGTTAC	This study, *Eco*RI
	YB-0083 (R)	GTCAGTCGTCGAC CATTAATATTGGTGAATGGG	This study, *Sal*I
Sp^r cassette amplification for pIMAY Δ*sec* and Δ*tst-1*	YB-0098 (F)	GTCAGTCGCGGCCGC CGAATGGCGATTTTCGTT	This study, *Not*I
	YB-0069 (R)	AGTCAGTCGAATTC CATGATTACGCCAAGCTCG	This study, *Eco*RI
Confirmation of pIMAY Δ*sec* and Δ*tst-1*	IM151 (F)	TACATGTCAAGAATAAACTGCCAAAGC	[31]
	IM152 (R)	AATACCTGTGACGGAAGATCACTTCG	[31]
Confirmation of Δ*sec* in *S. aureus*	YB-0123 (F)	AGGCAGGTACTTCGGTAC	This study
	YB-0107 (R)	TTAATTATCAATGGCAAG	This study
Confirmation of Δ*tst-1* in *S. aureus*	YB-0090 (F)	ACTGCAACACAGGGTGT	This study
	YB-0079 (R)	TAACGGGAATGTGCCTATAT	This study

replacement for the *sec* or *tst-1* gene. The 1228 bp cassette was amplified from the pDG1727 plasmid [30] with Phusion polymerase, 1 × Phusion GC Buffer. The cycling steps included initial denaturation at 98 °C for 3 min, 15 cycles of denaturation at 98 °C for 15 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 2 min, followed by another 20 cycles of denaturation at 98 °C for 15 s and annealing and elongation at 72 °C for 150 s, with a final extension step at 72 °C for 7 min. The sizes of the PCR products were confirmed using agarose gel electrophoresis, and later purified using the GeneJet PCR purification kit (Thermo Fisher Scientific, Waltham, MA, USA) for the digestion and ligation steps.

The pIMAY plasmid was used as a vector to construct the double recombination deletion mutants [31]. Upstream and downstream homologous fragments, and the antibiotic cassette containing restriction sites on both sides for each *sec* and *tst-1* deletion construct, were digested with corresponding FastDigest™ restriction enzymes (Thermo Fisher Scientific, Waltham, MA, USA). These fragments were then sequentially ligated to the pIMAY plasmid at a molar ratio of 1:3 and transformed into *Escherichia coli* DC10B cells. The constructs were confirmed by restriction analysis and agarose gel electrophoresis and are denoted pIMAYΔ*sec* and pIMAYΔ*tst-1*.

Electrocompetent *S. aureus* MAS106, MAS602 and MAS660 bacteria were prepared as described by Monk et al. and Löfblom et al. [31,32] and stored at −80 °C. The GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to isolate the pIMAYΔ*sec* and pIMAYΔ*tst-1* plasmids. A sufficiently high concentration of plasmid (>1 μg × μl^{-1}) to perform transformation of *S. aureus* was achieved by concentration using ethanol precipitation.

Competent bacteria of the wild type strains were thawed on ice for 5 min, allowed to rest for 5 min at room temperature, and then centrifuged for 1 min at 5000×*g* (Model 5424, Eppendorf, Hamburg, Germany). The cell pellet was suspended in 50 μl of a sterile 500 mM sucrose solution (Acros Organics, Geel, Belgium) supplemented with 10 % glycerol (Sigma-Aldrich, St. Louis, MO, USA). Five μg of the corresponding plasmid, in a maximum total volume of 5 μl, was added and mixed with the competent bacteria. Transformation was performed using 0.1 cm Gene Pulser electroporation cuvettes and the Gene Pulser equipment (both from Bio-Rad, Hercules, CA, USA) with settings of 21 kV × cm^{-1}, 100 Ω and 25 μF. Afterwards, 1 mL of tryptic soy broth (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 500 mM sucrose was added, and the transformation mixture was incubated for 2–3 h at 28 °C, 200 rpm. The bacteria were then plated on BHI agar containing 10 μg/mL chloramphenicol, and grown at 28 °C for 48 h.

After transformation, double recombination mutant selection was performed as described by Monk et al. [31]. Deletion of the *sec* and *tst-1* genes was confirmed by growth of the strain on BHI agar plates (Difco Laboratories) with 100 μg/mL spectinomycin (Sp^r 100), and its absence of growth on BHI agar with 10 μg/mL chloramphenicol. Further confirmation of the mutants was performed using PCR with the primer pair YB-0123-YB-0107 for Δ*sec* and YB-0090-YB-0079 for Δ*tst-1*, Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and Chelex-extracted genomic DNA from overnight cultures of the mutants as the template. The cycling steps included initial denaturation at 98 °C for 30 s, denaturation annealing and elongation (35 cycles) at 98 °C for 10 s, 50 °C for 20 s, and 72 °C for 2 min 30 s, followed by final elongation at 72 °C for 5 min. PCR products were purified, diluted, premixed with the appropriate primer and sequenced by Eurofins Genomics, Germany. From all three wild type strains Δ*sec* mutants were achieved while Δ*tst-1* mutants were obtained from two of the strains, MAS106 and MAS602. Double recombination was never successful in MAS660 despite numerous attempts and therefore no Δ*tst-1* mutant from this strain could be included and investigated in the study.

2.3. Growth characterization

Each wild type and mutant strain were resuscitated on BHI agar plates from −80 °C glycerol stock, and incubated overnight at 37 °C. A single colony from overnight plates was transferred and incubated in 20 mL BHI broth in a 50 mL Falcon tube overnight at 37 °C, 200 rpm (Innova® 40, Incubator Shaker Series, New Brunswick Scientific, Edison, NJ, USA). The overnight cultures were centrifuged at 3220×*g* (Model 5810R, Eppendorf, Hamburg, Germany) for 5 min and the supernatant was discarded. The pellet was washed twice with the same volume of 0.9 % NaCl (Merck Millipore, Darmstadt, Germany) to remove secreted toxins and metabolites from the overnight growth. The optical density at 620 nm (OD_{620}) was measured using a UV/visible spectrophotometer (Ultrospec 2100 pro, GE Healthcare, Little Chalfont, UK). Fresh bacterial BHI broth was inoculated with the washed preculture aiming at a starting OD_{620} of 0.1 in a 1-L baffled flask containing 200 mL BHI broth. Cultivation was performed at 37 °C and 200 rpm. The growth of all the wild type and mutant strains was determined by measuring the OD_{620} and viable counts. Samples were collected hourly between 0 and 9 h and at 24 h for viable count, OD_{620} and ELISA analysis. The viable count was determined using a 10-fold serial dilution of the culture. One hundred μl of the last two dilutions was plated on BHI agar in duplicate and incubated overnight at 37 °C before the colony forming units (CFU) were counted. The growth characterization experiments were performed in three biological replicates ($n=3$) for the wild type strains and two biological replicates ($n=2$) for the five deletion mutants. Maximum specific growth rate (μ max, h^{-1}) was calculated using linear regression by natural logarithm from four consecutive OD_{620} measurements for each replicate. Average value and standard deviation for each strain are presented in Table 4.

Table 4
Maximum specific growth rate (μ max h^{-1}) and maximum OD_{620} of MAS106, MAS602, and MAS660, and the isogenic *sec* and *tst-1* deletion mutants. Average value and standard deviation are presented for each strain.

Strain	μ max (h^{-1})	max OD_{620}
MAS106 ($n = 3$)	1.37 ± 0.07	12.07 ± 0.58
MAS106Δ*sec* ($n = 2$)	1.17 ± 0.05	15.05 ± 0.07
MAS106Δ*tst-1* ($n = 2$)	1.20 ± 0.09	11.8 ± 1.70
MAS602 ($n = 3$)	1.34 ± 0.09	11.03 ± 0.36
MAS602Δ*sec* ($n = 2$)	0.95 ± 0.07	13.18 ± 3.92
MAS602Δ*tst-1* ($n = 2$)	1.05 ± 0.45	11.08 ± 0.88
MAS660 ($n = 3$)	1.27 ± 0.18	11.16 ± 0.81
MAS660Δ*sec* ($n = 2$)	1.15 ± 0.01	12.5 ± 0.35

2.4. Toxin quantification by ELISA

To measure the production of SEC and TSST-1 toxins during growth, an in-house ELISA was set up using antibodies from Toxin Technology (Sarasota, FL, USA) according to the revised laboratory protocol for staphylococcal enterotoxin A described by Wallin-Carlquist et al. [33] The absorbance was measured at 405 nm with a Multiskan Ascent spectrophotometer (Electron Corporation, Thermo Fisher Scientific). Standard curves using two times serial dilutions from 10 ng/mL to 0.039 ng/mL in BHI were obtained for SEC and TSST-1 using highly purified SEC and TSST-1 toxins (Toxin Technology). Measured absorbance values for standard curve samples (average of triplicate wells) were plotted against standard toxin concentrations. Based on absorbance value of each experimental sample the concentration of SEC or TSST-1 toxin was then calculated using the corresponding standard curve. The concentrations were calculated using the linear regression equation and are expressed as ng/mL of toxin. For each biological replicate of the wild type or mutant strains, three technical replicates of ELISA measurements were performed. If required, the experimental samples were diluted appropriately in relation to the linear range of the respective standard curve (ranging between 5 ng/mL to 0.16 ng/mL). The quantification limit was at 0.31 ng/mL for each respective toxin.

2.5. Cell culture infection experiment

BME-UV cells [34] were used as a model to investigate the infection capability of both wild type strains and deletion mutants. All the bacterial substrates and media used were produced at the Swedish Veterinary Agency (SVA), Uppsala, Sweden. The cells were grown in 6-well cell culture plates (Sarstedt, Nümbrecht, Germany). *S. aureus* wild type and mutant strains were harvested from overnight cultures on 5 % bovine blood agar and BHI Spc100, respectively (both from Oxoid, Hampshire, UK). Bacteria were diluted with Ham's F12 cell culture medium 40 %, RPMI 1640 30 %, NCTC-135 medium 20 %, FCS 10 %, lactose 0.1 %, lactalbumin hydrolysate 0.1 %, GSH 1.2 mM, L-ascorbic acid 10 μg/mL, hydrocortisone 1 μg/mL, insulin 1 μg/mL, prolactin 1 μg/mL and gentamycin 50 μg/mL, to a final concentration of approximately 10^6 CFU/mL. The growth medium used for the BME-UV cells was removed and 3 mL of 10^6 CFU/mL from one of the bacterial strains was added per well to initiate infection. After 2 h of incubation at 37 °C with 5 % CO_2, the supernatants were removed and the BME-UV cells were washed twice with 3 mL PBS with calcium and magnesium, pH 7.4. To kill the remaining extracellular *S. aureus*, 3 mL of cell culture medium containing 100 μg/mL gentamicin was added, and the cells were incubated at 37 °C with 5 % CO_2 for 2 h. The BME-UV cells were then washed twice with 37 °C PBS, and incubated for another 3 h at 37 °C with 5 % CO_2 in culture medium without antibiotics.

After incubation, the BME-UV cells were washed twice with 37 °C PBS and lysed by the addition of 1 mL ice-cold Milli-Q water for 1 min. The BME-UV cells were then scraped from the 6-well plates. An aliquot of each lysate was diluted 10-fold in medium and plated onto 5 % bovine blood agar or BHI Spc100 agar. The plates were incubated overnight at 37 °C. The number of viable intracellular *S. aureus* was calculated and expressed as CFU/mL. Remaining BME-UV cell lysates were stored at −80 °C for subsequent proteomic analysis. Two independent experiments (three replicates per strain and experiment) were performed using MAS106, MAS106Δ*sec*, MAS106Δ*tst-1*, MAS660 and MAS660Δ*sec*, and two independent experiments (three replicates per strain in first experiment and six replicates per strain in second experiment) were performed using MAS602, MAS602Δ*sec* and MAS602Δ*tst-1*. For each strain the infection ratio was calculated as the average number of viable intracellular *S. aureus* per strain, in relation to the initial infection dose.

2.6. Proteomic analysis

Proteomic analyses were performed on lysates of BME-UV cells infected with *S. aureus* wild type strains and deletion mutants. Lysates of non-infected BME-UV cells (Mock) were also analyzed. Five biological replicates were analyzed for MAS 602 and four biological replicates were analyzed for MAS 106 and MAS 660, respectively. The BME-UV cell lysates from each sample were centrifuged at 10,000×*g* for 5 min at 4 °C. The supernatant was concentrated using Amicon® Ultra-0.5 mL Centrifugal Filter with Ultracel-10 membrane (Merck Millipore, Darmstadt, Germany). Both the concentrated supernatant and pellet were treated with 100 μL lysis buffer (20 mM HEPES, 9 M urea, one tablet of Complete™ Mini EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, Mannheim, Germany)), combined in one tube, which was then subject to sonication for 1 s, 10 times. The tube was subsequently centrifuged at 14000 × g for 5 min, and the supernatant was transferred to a new tube. Proteomic analyses were performed at the MS-Based Proteomics Facility, Department of Chemistry – BMC, Uppsala University (Uppsala, Sweden).

Samples were dissolved in sodium dodecyl sulfate lysis buffer (4 % SDS in 100 mM Tris/HCl pH 7.6) at 70 °C for 5 min. Solubilized proteins were separated from sample debris by centrifugation at 16,000 g for 5 min. The total protein concentration in the samples was measured using the Bradford protein assay with bovine serum albumin (BSA) as standard. Aliquots corresponding to 20 μg protein were taken out, reduced, alkylated and in-solution digested by trypsin according to a standard operating protocol. The samples were purified by Pierce C18 Spin Columns (Thermo Scientific), dried and redissolved in 55 μL 0.1 % Formic acid.

Peptides were separated using an Easy Nano Flow System (Thermo Fisher Scientific) on a Pierce C18 column (75 μm ID, 3 μm C18-beads) (Thermo Fisher Scientific), fitted with a pre-column (100 μm ID, 5 μm C18-beads) (Thermo Fisher Scientific), using a linear gradient from 4 % to 48 % acetonitrile with 0.1 % formic acid for 131 min, followed by 75 % acetonitrile for 10 min and 4 % acetonitrile for 9 min for re-equilibration. The flow rate was 250 nL/min. Peptides were ionized using nano-electrospray ionization and analyzed in full scan mode (m/z = 400–1750) on an QE Plus Orbitrap (Thermo Fisher Scietific) operated at a resolving power of 60,000 with accumulation of 1,000,000 ions. MS/MS spectra were acquired using data-dependent acquisition, with the 10 most intense peaks being isolated and fragmented in the linear ion trap using high-energy dissociation (HCD).

2.7. Proteomic data analyses

MS data were analyzed using Proteome Discovery 2.1.0.81, supplied by Thermo Scientific, to identify the total number of proteins. MaxQuant 1.5.6.5, supplied by the Max Plank institute, was used to quantify the proteins and the Andromeda search engine to match MS/MS spectra to peptide sequences derived from the Uniprot bovine database [35]. The datasets from the infected cells were normalised to the non-infected cells. The following parameters were used for data processing:

maximum of two miss cleavages, mass tolerance of 4.5 ppm for main search, trypsin as digesting enzyme, carbamidomethylation of cysteins as fixed modification, and the oxidation of methionine and acetylation of the protein N-terminus as variable modifications. At least two peptides, out of which at least one is unique, composed of a minimum of 7 amino acids, were required for protein identification.

Protein data were analyzed using principal component analysis (PCA; prcomp, stats package) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) using the ropls package. Significantly altered proteins were identified based on variable influence on projection (VIP) for the predictive component (VIP_{pred}), with a conservative threshold of one to identify the most influential proteins [36].

3. Results

3.1. DNA sequencing and confirmation of deletion mutants

Primarily, DNA sequence analyses of the *sec* and *tst-1* genes in MAS106, MAS602 and MAS660, were performed. The results showed that the *sec* and *tst-1* sequences respectively in all three wild type strains were identical. Based on the sequencing results, genetic constructs were designed to create deletion mutants by double homologous recombination for *sec* and *tst-1*. Complete replacement of the *sec* or *tst-1* gene with a Sp^r antibiotic cassette was confirmed by sequencing of the resulting deletion strains, denoted MAS106Δ*sec*, MAS106Δ*tst-1*, MAS602Δ*sec,* MAS602Δ*tst-1* and MAS660Δ*sec*. No deletion mutant was obtained for MAS660Δ*tst-1* despite numerous attempts.

3.2. Growth characterization

The growth in BHI broth of wild type and mutant strains was studied over 24 h and compared using OD_{620} and viable counts. The maximum specific growth rate (μ max h^{-1}) of all strains based on the natural logarithm of obtained OD_{620} values ranged from 0.95 ± 0.07 to 1.37 ± 0.07 (Table 4).

3.3. Production of SEC and TSST-1 toxins

The production of SEC and TSST-1 toxins in wild type strains was monitored regularly during growth for 24 h (Fig. 1). No SEC or TSST-1 was formed by the respective isogenic mutants, confirming complete deletion of the gene. Deletion of the *sec* gene did not affect the formation of TSST-1, nor did deletion of the *tst-1* gene affect the formation of SEC (Fig. 2). At 24 h, the accumulated amount of TSST-1 formed by all wild type strains and Δ*sec* mutants were noticeably higher, about 10 times, than the accumulated amount of SEC produced at 24 h. MAS106 formed the lowest amount of SEC at 24 h. At this time, MAS602 and MAS660 showed similar SEC concentrations (1664.5 ± 250.3 ng/mL and 1559.2 ± 91.3 ng/mL, respectively), while the SEC concentration formed by MAS106 was seven to eight times lower (212.2 ± 45.5 ng/mL). A similar finding was observed in the TSST-1 concentration at 24 h. MAS660 and MAS602 formed 26387 ± 3576.6 ng/mL and 32745.4 ± 11249.3 ng/mL of TSST-1, respectively, while MAS106 formed 3592.2 ± 1348.4 ng/mL TSST-1, i.e., seven to nine times lower than the other two.

3.4. BME-UV cell culture infection

The average concentration of the infection solution was for MAS602, MAS602Δ*sec* and MAS602Δ*tst-1*, 4.2 x 10^6/mL, 8.3 x 10^6/mL and 1.5 x 10^7/mL, respectively. The corresponding concentrations for MAS106, MAS106Δ*sec*, MAS106Δ*tst-1* were 5.4 x 10^5/mL, 6.3 x 10^5/mL and 3.6 x 10^6/mL, respectively and for MAS660 and MAS660Δ*sec* they were 7.1 x 10^6/mL and 1.3 x 10^7/mL, respectively. The infection capabilities of the mutants were compared to those of the corresponding wild type strains. The lower infection ratios for all *sec* deletion mutants, MAS602Δ*sec*, MAS106Δ*sec* and MAS660Δ*sec,* were more pronounced and consistent in the two independent experiments compared to the *tst-1* deletion mutants, MAS106Δ*tst-1*, and MAS602Δ*tst-1,* in relation to respective wild type strain (Fig. 3). The lowest infection capability was observed in MAS602Δ*sec*.

3.5. Proteomics analysis

The proteome of BME-UV cells infected with the wild type strains and the isogenic mutants as well as the non-infected BME-UV cells (Mock samples) were examined using PCA. Initial analysis revealed the first component of the PCA to reflect the total protein level and this component was consequently removed from the analysis. For MAS602 and MAS660, a clear clustering of non-infected and WT-infected cells was observed in the PCA score scatter plot, suggesting *S. aureus* infection to elicit a systematic alteration in the BME-UV cell proteome (Fig. 4). For MAS106 no obvious clustering was observed in the PCA analysis and this strain was therefore excluded from further analysis by OPLS-DA, to avoid overfitting [37]. Both MAS602Δ*sec* and MAS660Δ*sec* infected BME-UV cells showed a proteome that differed from the WT-infected cells ($p = 0.096$ and $p < 0.01$, respectively), being more similar to the non-infected cells. Next, OPLS-DA was performed to characterize changes in the BME-UV cell proteome elicited by infection by the WT strains of MAS602 and MAS660. These analyses revealed 693 proteins to be altered by MAS660 and 432 proteins by MAS602. Out of these, 76 were altered after infection by both strains out of which 36 were up- or down-regulated in the same direction (Table 5). The remaining 40 proteins were altered in opposite directions (Supplementary material).

4. Discussion

S. aureus is known to be a common cause of bovine mastitis [38,39]. Both the *sec* and the *tst-1* genes are frequently detected in *S. aureus* strains isolated from dairy cows with mastitis [12–18]. There are several indications that these toxins are important in the *S. aureus* infection process [23–25]. The genes encoding SEC and TSST-1 are often located

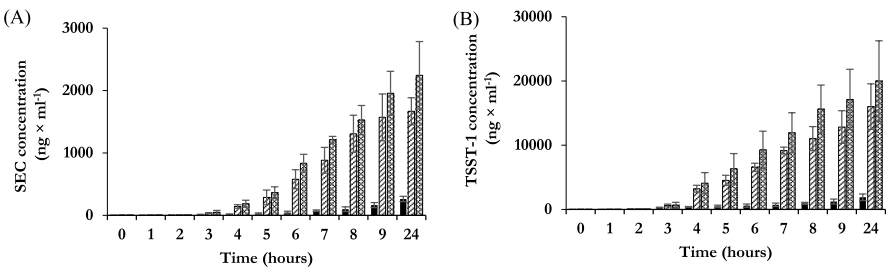

Fig. 1. SEC and TSST-1 concentrations of MAS106, MAS602, and MAS660 in BHI medium at 37 °C, 200 rpm for 24 h. (A) SEC and (B) TSST-1. MAS106 (■), MAS602 (▨), MAS660 (▩). Average values and standard deviations are based on three biological replicates and three technical replicates for each biological replicate.

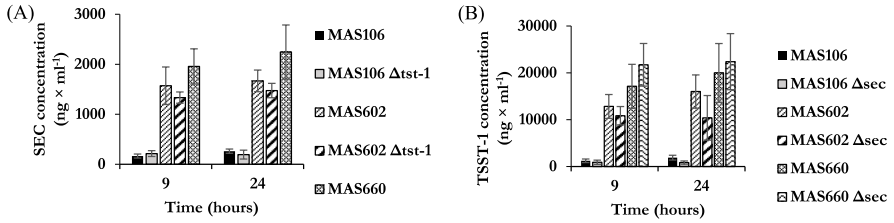

Fig. 2. SEC and TSST-1 concentration of MAS106, MAS602, MAS660 and the isogenic *sec* and *tst-1* deletion mutants in BHI medium at 9 and 24 h. Culture was grown at 37 °C, 200 rpm for 24 h. (A) SEC and (B) TSST-1. The concentrations of SEC in the *sec* mutants (MAS106Δ*sec*, MAS602Δ*sec,* and MAS660Δ*sec*) were below the detection limit, as was the TSST-1 concentration produced by the *tst-1* deletion mutants (MAS106Δ*tst-1*, and MAS602Δ*tst-1*). Average values and standard deviations are based on three biological replicates for the wild type strains and two biological replicates for the mutant strains. Three technical replicates were performed for each biological replicate.

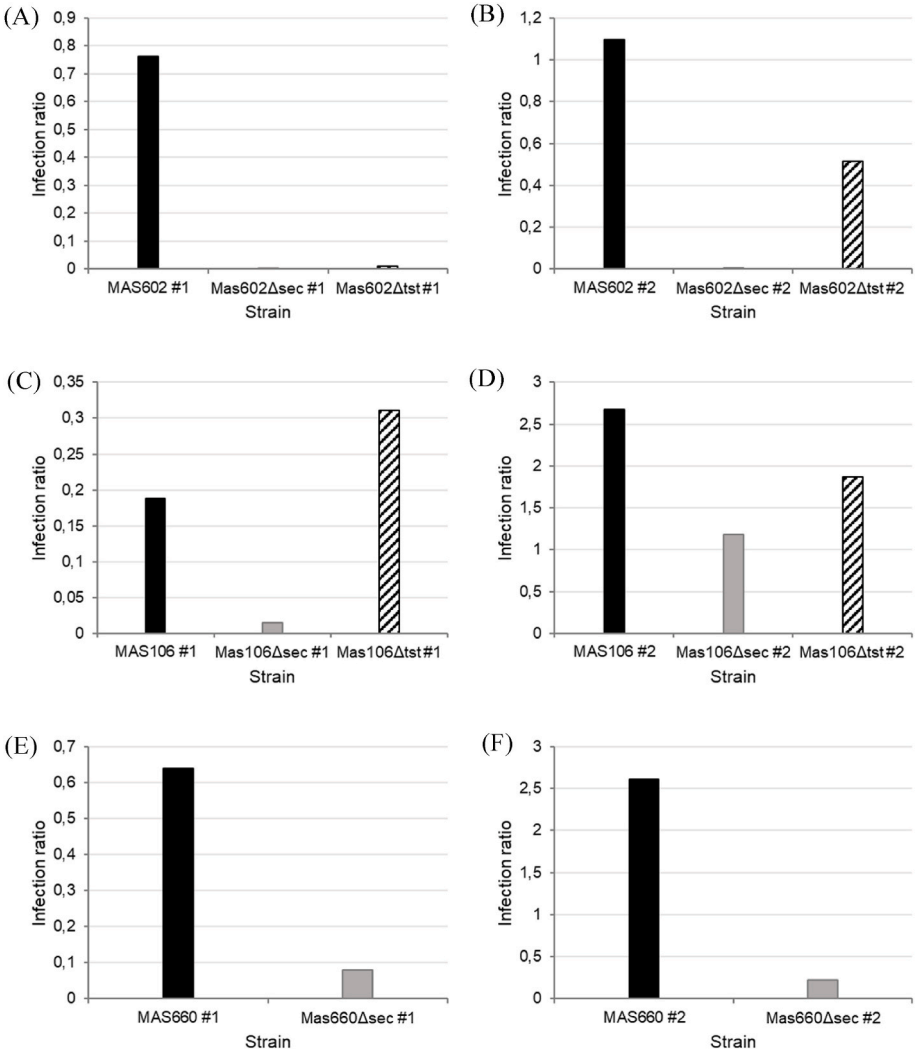

Fig. 3. Infection capability of MAS602 (A and B), MAS106 (C and D), MAS660 (E and F) and the isogenic *sec* and *tst-1* deletion mutants, tested in BME-UV cells. Infection capability was calculated by the ratio of the average number of viable intracellular *S. aureus* per strain, in relation to the initial number of *S. aureus* inoculated into the BME-UV cells. Total incubation time after infection was 7 h. Two independent experiments (#1 and #2 respectively: three replicates per strain and experiment) were performed using MAS106, MAS106Δ*sec*, MAS106Δ*tst-1*, MAS660 and MAS660Δ*sec*, and two independent experiments (#1 and #2 respectively: three replicates per strain in first experiment and six replicates per strain in second experiment) were performed using MAS602, MAS602Δ*sec*, and MAS602Δ*tst-1*.

on the same pathogenicity island making it difficult to study the effects of the two toxins separately. However, Kuroishi et al. (2003) have demonstrated that intramammary inoculation in the bovine udder of SEC, but not TSST-1, induce a significant increase in somatic cell counts, indicating that SEC rather than TSST-1 plays an important role in the pathology of staphylococcal bovine mastitis [16]. Seeking further evidence for the potential roles and action of SEC and TSST-1 we investigated the infection capability of three *S. aureus* strains isolated from

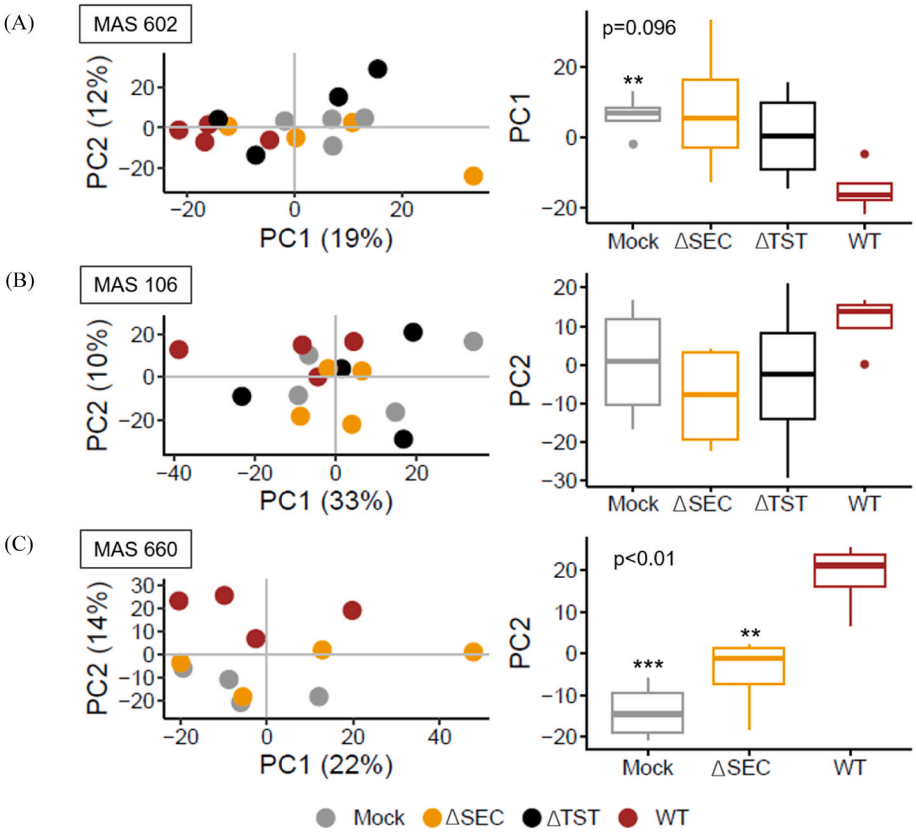

Fig. 4. Score plot from principal component analysis of the proteome in BME-UV cells infected with *S. aureus* (A) MAS602WT (red dot), MAS602Δ*sec* (yellow dot), MAS602Δ*tst-1* (black dot); (B) MAS106WT, MAS106Δ*sec*, MAS106Δ*tst-1* and (C) MAS660WT, MAS660Δ*sec* in relation to non-infected BME-UV cells denoted Mock (grey dot). Five biological replicates were analyzed for MAS602 and four biological replicates were analyzed for MAS106 and MAS660, respectively.

bovine clinical mastitis, which were deleted for *sec* and *tst-1*, respectively. *S. aureus* can be taken up by cells that normally are not non-phagocytic, like mammary epithelial cells. In the host cell, the bacteria are protected against attacks from extracellular host defence mechanisms and resistant to antibiotic therapy [40]. In our system to study the infection capability of *S. aureus* we found a reduced invasion to varying extent for the Δ*sec* deletion mutants in bovine mammary cells in vitro. This reduction in infection ratio was further to some extent supported by the results of the proteomic profile analyses, where two of the three Δ*sec* deletion mutant strains, MAS602Δ*sec* and MAS660Δ*sec*, produced a difference in the proteome of the infected BME-UV cells compared to infection with the corresponding wild type strains. Several differentially expressed proteins in *S. aureus* infected mammary epithelial cells, like heat shock proteins, glutathione reductase and RNA-binding proteins, suggest alterations in stress response and post-transcriptional regulation. The heat shock proteins can affect the peptide synthesis under stress intracellularly (Accession F1MUZ9, UniProt.org) or they can act extracellularly by modulating the immune response [41]. Thus, one can speculate that heat shock protein can support or is supported by SEC formation in infected mammary epithelial cells. In mastitis the clinical manifestation of the infection might even partly be mediated by the immunomodulatory effects of heat shock proteins. Glutathione reductase serves in maintaining proper cell function [42]. The downregulation of this protein might be a factor contributing to the death of infected cells and the release of bacteria able to infect new cells. These findings strengthen the hypothesis that SEC is of importance in the infection process of *S. aureus* into mammalian cells even though its precise role still remains unknown and subject for further investigations.

Regarding TSST-1, the results were less clear, but a role of TSST-1 cannot be ruled out. In our system there was a reduced infection capability with the MAS602Δ*tst* compared to wild type MAS602, while there was not such an obvious equivalent effect for MAS106Δ*tst*. The observed effects are probably not caused by differences in bacterial growth characteristics, since growth rates of the wild type strains and the deletion mutants were similar when grown in BHI medium. This can be expected considering that neither the SEC nor the TSST-1 toxin has any, to our knowledge, known essential function in cell replication, growth or viability. The accumulation of TSST-1 during growth was about 10 times higher than that of SEC in all wild type strains. These two toxins are often genetically located close to each other in the $SaPI_{bov}$ backbone but *sec* can also be located on a plasmid [20,43]. SEC and TSST-1 are both known to be positively regulated by the same Agr quorum sensing system but also other regulatory mechanisms have been reported for TSST-1 [44–46]. These differences in genetic localization and regulation might also play a role for their potential and different involvement during infection and it is indeed interesting and important to acknowledge and study further.

Interestingly, the ability of MAS106 to form SEC and TSST-1 was 7–8 times lower than those of MAS602 and MAS660. The reason for this is not yet known, but differences in the DNA sequences encoding the toxins can be ruled out as DNA sequence analysis showed no differences in the *sec* and *tst-1* gene sequences in these three strains. Earlier analyses of the three studied *S. aureus* strains show that they all are regulated by *agr* II. In addition, other regulatory genes in *S. aureus*, such as staphylococcal accessory regulator A *(sarA)* and the gene for histidine protein kinase were the same in the three strains [28]. Still, MAS106 belongs to another ST than MAS602 and MAS660 and there might be regulatory mechanisms, located in other positions of the genome that differs between the STs. Similar variability between strains in producing toxin has also been observed for other enterotoxins such as enterotoxin A and enterotoxin D [47,48]. Furthermore, for MAS 106 no clear alteration of the proteome

Table 5
Up- and downregulated proteins identified by OPLS-DA in BME-UV cells infected by *S. aureus* WT strains MAS602 or MAS660.

Up-regulated proteins		Down-regulated proteins	
UniProt identifiers	Protein name	UniProt identifiers	Protein name
A6QLZ0	Galectin	A5D7Q4	CSDA protein
ANXA8	Annexin A8	A6QP73	ATP-dependent RNA helicase
E1BM93	Retinol dehydrogenase 11	A7MB38	SFRS4 protein
E1BPV2	Alkylglycerone-phosphate synthase	DHPR	unknown protein
F1MUZ9	60 kDa heat shock protein, mitochondrial	E1BEX4	Uroporphyrinogen decarboxylase
K1C19	unknown protein	E1BKJ7	Insulin-like growth factor 2 mRNA-binding protein 1 (IGF-II)
MCM7	DNA replication licensing factor MCM7	E1BKZ1	Glutathione reductase
P13696	Phosphatidylethanolamine-binding protein 1 (PEBP-1)	ELOC	Elongin-C (EloC)
PPIB	Peptidyl-prolyl cis-trans isomerase B (PPIase B)	ERH	Enhancer of rudimentary homolog
Q0VCS3	UBQLN1 protein	F1MMK8	Lysine-tRNA ligase
Q3ZBX0	Basigin	F1MS38	Protein NDRG1
RACK1	Small ribosomal subunit protein RACK1	KCRU	unknown protein
RS20	unknown protein	MOES	Moesin
RS3	unknown protein	Q29RQ2	KH RNA binding domain containing, signal transduction associated 1
RS7	unknown protein	Q3SYT5	Pinin
S10AA	unknown protein	RBM8A	RNA-binding protein 8A
TAGL2	unknown protein	STRN3	Striatin-3
		TBB6	unknown protein
		TEBP	unknown protein

of BME-UV cells was observed.

The failure to obtain a MAS660Δ*tst-1* deletion mutant despite numerous attempts could possibly also be linked to the overall pronounced variability observed across clinical isolates in combination with the robust restriction-modification (RM) systems active in *S. aureus* that also present implications for genetic engineering [49,50]. Strain-specific variation of the restriction-modification systems could serve as one plausible explanation for not being able to generate a MAS660Δ*tst-1* deletion mutant [51].

The demonstrated role of especially SEC in the infection of bovine mammary epithelial cells supports the earlier findings that the manifestation of the infection is affected by the virulence factors of the infecting *S. aureus* strain. Different strain types elicit different immune responses, both regarding degree of response and timely [52,53]. In Murphy et al. (2019), strains belonging to CC151 tended to be less immunogenic than strains belonging to CC97, which is another common strain type found in milk from bovine mastitis in Sweden [27]. Both these strain types are commonly found in bovine mastitis all over the world [54].

In an experimental infection in dairy cows, the *S. aureus* strain type eliciting a low immune response resulted in persistent mastitis, while the more immunogenic strain type resulted in a non-persistent mastitis [55]. About 26 % of *S. aureus* stains isolated from acute clinical mastitis in Sweden have the genes *sec* and *tst* (pers. com. Mikael Leijon; unpublished results). The strongest evidence we have found in literature for SEC having a role in mastitis is that by Fang et al. (2019), who demonstrated that SEC caused severe damage of the mouse mammary gland which could be neutralized by anti-SEC antibodies. Immunization of cows with different variants of SEC has also been shown to impact mastitis, resulting in a lower somatic cell count in milk from vaccinated cows after challenge with *S. aureus*, compared to non-vaccinated control cows [24,25]. No vaccination experiments employing TSST-1 in the context of mastitis could be found in the literature. However, there are reports on the use of inactivated TSST-1 in relation to toxic shock syndrome. In one study, the immunization of mice with inactivated TSST-1 resulted in a protective effect against systemic infection and colonization by *S. aureus* [26]. Several virulence factors contribute to the invasion of *S. aureus* into mammary cells. Interestingly, a positive correlation has been observed between the cumulative number of virulence genes in *S. aureus* isolates and the severity of bovine mastitis [52]. In the same study pyrogenic toxin superantigens were demonstrated in a significantly higher prevalence among *S. aureus* isolated from persistent intramammary infections compared to nonpersistent infections and *sec* and *tst* were strongly associated to a *S. aureus* strain type that predominantly were found in severe cases of clinical mastitis. If it turns out that infections with *S. aureus* with *sec* and *tst-1* are more severe than infections with strains without these genes, there is a potential/incentive for interventions to more actively avoid or fight infections with strains causing more severe disease.

The current study has certain limitations that should be acknowledged. Firstly, a MAS660Δ*tst-1* deletion mutant is missing in this study. Secondly, although the deletion mutants were verified by both PCR and sequencing of the insertion sites to confirm correct replacement of the *sec* or *tst-1* genes with the Sp^r antibiotic resistance cassette at the intended genomic locus, the possibility of ectopic integration of the deletion construct elsewhere in the genome has not been formally excluded. Whole genome sequencing would be the preferred approach to assess this, but this was not feasible within the scope of the present study. Future work should include such analyses to further validate these mutant strains. Thirdly, the proteomic study was under-powered and as such it was not possible to demonstrate any detailed conclusion on the relationship between the presence/absence of *sec* and *tst-1* genes and resulting altered proteome in the infected BME-UV cells. It is not possible to elucidate whether the observed response is a direct consequence of infection by wild type and toxin deletion mutant strains of *S. aureus* or a more general stress response of infection. This is open for further investigations.

5. Conclusions

Three *S. aureus* strains, isolated from acute clinical cases of bovine mastitis in Sweden, have been investigated. Although growth rates of these strains were similar, their ability to form the two toxins SEC and TSST-1 varied. The decrease in infection capability and the altered proteome of BME-UV cells when infected with *sec* gene deletion mutant strains show that SEC can play a role in the infection process. Our results support the findings of previous vaccination studies [24–26] indicating that *S. aureus* toxins may play a role in the infection and colonization process, and can be interesting targets for the development of new strategies to prevent and treat bovine mastitis. This is the first study that has generated *S. aureus* deletion mutants in clinical isolates for the purpose of studying the possible roles of SEC and TSST-1 in the infection of bovine mammary epithelial cells. The methodological approach of investigation infection capability using wild type and isogenic mutants along with proteome analysis is promising and should be explored further. Additional investigations of the proteins affected and the biochemical pathways they follow will be crucial in improving our understanding of the role of *S. aureus* toxins in infection.

CRediT authorship contribution statement

Yusak Budi Susilo: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Karin Artursson:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Lihong Liu:** Methodology, Investigation, Formal analysis, Data curation. **Julia Södergren:** Writing – review & editing, Formal analysis, Data curation. **Peter Spégel:** Writing – review & editing, Validation, Formal analysis. **Jonas Bergquist:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis. **Jenny Schelin:** Writing – review & editing, Validation, Supervision, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2025.107891.

Data availability

Data will be made available upon request.

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