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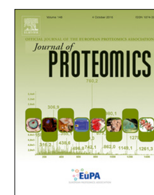

Comparative exoproteome profiling of an invasive and a commensal *Staphylococcus haemolyticus* isolate

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ABSTRACT

Staphylococcus haemolyticus is a skin commensal emerging as an opportunistic pathogen. Nosocomial isolates of *S. haemolyticus* are the most antibiotic resistant members of the coagulase negative staphylococci (CoNS), but information about other *S. haemolyticus* virulence factors is scarce. Bacterial membrane vesicles (MVs) are one mediator of virulence by enabling secretion and long distance delivery of bacterial effector molecules while protecting the cargo from proteolytic degradation from the environment. We wanted to determine if the MV protein cargo of *S. haemolyticus* is strain specific and enriched in certain MV associated proteins compared to the totalsecretome.

The present study shows that both clinical and commensal *S. haemolyticus* isolates produce membrane vesicles. The MV cargo of both strains was enriched in proteins involved in adhesion and acquisition of iron. The MV cargo of the clinical strain was further enriched in antimicrobial resistance proteins.

Data are available via ProteomeXchange with identifier PXD010389.

Biological significance: Clinical isolates of *Staphylococcus haemolyticus* are usually multidrug resistant, their main virulence factor is formation of biofilms, both factors leading to infections that are difficult to treat. We show that both clinical and commensal *S. haemolyticus* isolates produce membrane vesicles. Identification of staphylococcal membrane vesicles can potentially be used in novel approaches to combat staphylococcal infections, such as development of vaccines.

1. Introduction

Staphylococcus (S) haemolyticus is a skin commensal which has gained increased attention as an opportunistic pathogen, particularly in patients with reduced immune defence and implanted medical devices. Coagulase negative staphylococci (CoNS) are a leading cause of sepsis, and *S. haemolyticus* is the second most frequently isolated CoNS from blood culture after *Staphylococcus epidermidis* [1]. Nosocomial isolates of *S. haemolyticus* are the most antibiotic resistant members of the coagulase negative staphylococci (CoNS) [2], but little is known about other factors involved in the transition from a "benign" skin commensal to an invasive lifestyle.

Secreted and cell surface expressed bacterial proteins are known to be major determinants of virulence and pathogenicity, and are the first to interact with host cells [3]. In *Staphylococcus aureus* which has a plethora of virulence factors and immune evasive factors, secreted proteins interact with the host immune defence in several ways [4]. CoNS are less virulent in comparison to *S. aureus*, but the phenol soluble modulins (PSMs) of several CoNS have been described as important virulence factors promoting sepsis and biofilm development [5]. Recently a novel α-type PSM with pronounced cytolytic capacity was identified in addition to the three known PSMβ1-3 in *S.haemolyticus* culture filtrates [6].

Another mediator of bacterial virulence is membrane vesicles

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(MVs). Although MVs have been thoroughly described in Gram-negative bacteria, they were only recently discovered in Gram-positive bacteria [7,8]. MVs are spherical bi-layered structures of 20–100 nm that are produced and released during bacterial growth. The MVs enable secretion and long distance delivery of bacterial effector molecules while protecting the cargo from proteolytic degradation from the environment. Proteomic and functional analyses have revealed that MVs are enriched in proteins involved in virulence, antimicrobial resistance, microbe-host interaction and inter- bacterial communication, they can also act as decoys, thereby protecting the bacteria against host immunity and phage infections [9–11]. Both in *S. aureus* and in a sepsis strain of *S. epidermidis*, MVs were enriched in proteins characterized as virulence factors [12,13].

In this study we show for the first time that *S. haemolyticus* produces MVs. We further perform a comparative analysis of the MV cargo and the total secretome of a commensal and clinical isolate with respect to identification of strain specific and MV associated proteins. Previous studies did not perform a comparative analysis between the MV cargo and the total secretome. The results obtained through this study provide information about *S. haemolyticus* strain specific protein expression, which might reflect differences in the genetic background and gene expression of a clinical blood culture isolate and a skin isolate.

2. Materials and methods

2.1. Bacterial strains and culture conditions

One clinical *S. haemolyticus* blood culture isolate from a neonatal sepsis episode at a Paediatric ward at a Norwegian hospital, was used. This isolate, 51–08 is Methicillin resistant and also resistant to gentamicin, fusidic acid, macrolides, tetracycline and fluoroquinolones. The strain has been sequenced, and the sequence is deposited at the European Nucleotide Archive with the number (7067_4_39, ERS066281) [14]. The *S. haemolyticus* commensal isolate 57-1 was isolated from swabbing the armpit of a healthy adult, not exposed to antibiotics during the three previous months prior to sampling. The commensal strain, 57-1 was sensitive to all 11 antimicrobial agents tested [2]. Strain 57-1 is also sequenced, but the sequence is not yet published. The bacteria were picked from blood agar plates, and grown overnight in Tryptic Soy Broth (TSB, Becton Dickinson, Franklin Lakes, USA) at 37 °C. Overnight cultures were diluted 1:100 in 100 ml TSB (for isolation of proteins released by the bacteria into the supernatant) or in 1000 ml TSB (for isolation of membrane vesicles) and grown to OD_{600} of 2.0 ± 0.2.

2.2. Isolation of MVs from culture supernatants

Bacterial cultures at OD_{600} of 2.0 ± 0.2 were pelleted by centrifugation (5000 *g*, for 20 min, at 4 °C), and the supernatant was filtered through a 0.22 µm polyethersulfone membrane (Millipore express plus, Merck Millipore, Burlington, USA), before being concentrated using Amicon Ultra-15, 100 kDa, centrifugation units (Millipore express plus, Merck Millipore, Burlington, USA). Membrane vesicles were isolated by ultracentrifugation at 164, 326 × *g* (40. 000 rpm, using SW 60 Ti rotor from Beckman Coulter Brea, USA) for 2 h at 4 °C. The pellet was re-suspended in ice-cold Phosphate buffer saline (PBS) and further purified by gradient centrifugation. Thereto, the samples were mixed with OptiPrep TM (Sigma-Aldrich, Steinheim, Germany) to a final concentration of 30%, loaded to the bottom of a tube and further layered with 2000 µl 25% and 1000 µl 5% OptiPrep TM. The MV fractions were separated by density gradient centrifugation at an average of 84, 168 x g (30. 000 rpm, using SW 50 Ti rotor from Beckman Coulter, Brea, USA) for 3 h at 4 °C. Fractions were then collected from the top. The presence of proteins in the different fractions was determined by SDS-PAGE followed by Coomassie blue staining (BioRad, Hercules, USA). OptiPrep fractions were analysed by transmission electron microscopy (TEM) for confirmation of MV content and sample purity. Further, fractions containing MVs were pooled and diluted in 4 ml PBS, before centrifugation using a Vivaspin 4 turbo tube (Sartorius, Göttingen, Germany) at 5000 x g for 30 min in order to remove residual OptiPrep solution. The sterility of the isolated MV samples was verified by streaking small aliquots on blood agar plates followed by incubation at 37 °C overnight and further examined for growth of bacteria on the blood agar plate. All experiments were performed as three independent biological replicates, performed at three independent time points, using three independent starting cultures.

2.3. Transmission electron microscopy and atomic force microscopy analysis

MV samples were prepared for transmission electron microscopy (TEM) analyses using a standard negative stain method. 5 µl of the MV sample was applied to a formvar coated 75 mesh hexagonal copper grid. The grids were washed four times with ddH_2O and transferred to a 0.3% solution of uranyl acetate in 1.8% methylcellulose. The grids were then left to dry before microscopy. Electron micrographs were recorded using a JEOL JEM1010 microscope (Akishima, Japan), at 80 kV acceleration voltage.

For atomic force microscopy (AFM) analysis of whole cells of *S. haemolyticus,* the strain was grown overnight on Luria Bertani (LB) agar plates.

Sample preparation was carried out as described previously [15]. Representative images were collected by a nanoscope V atomic force microscope (Bruker AXS, Billerica, USA).

2.4. Protein precipitation and sample preparation for mass spectrometry

Proteins from culture filtrates and pooled MV fractions were precipitated and sample preparation was performed as described previously in [16]. Protein concentration was determined using the Qubit TM protein quantification kit (Thermo Fisher Scientific, Waltham, USA). Four µg of protein was reduced and alkylated with dithiothreitol and iodoacetoamide, respectively, prior to digestion with a 1:20 ratio of trypsin (V511A, Promega, Madison, USA). The resulting peptide mixture was purified and desalted using OMIX C18 tips (Varian, Inc., Palo Alto, USA) and dried in a speed vacuum centrifuge. Dried peptides were dissolved in 0.2% formic acid.

2.5. Protein identification and analysis by mass spectrometry

Mixtures of 2 µg peptides in 0.2% formic acid were loaded onto a Thermo Fisher Scientific EASY-nLC1000 system with an EASY-Spray column (C18, 2 µm, 100 Å, 50 µm, 50 cm). Peptides were fractionated using a 2–100% acetonitrile gradient in 0.1% formic acid over 50 min at a flow rate of 250 nl/min. The gradient had four steps; an 8 min gradient step from 4 to 8% acetonitrile followed by a 50 min step from 8 to 40% acetonitrile, a 8 min step to 100% acetonitrile and a final 8 min step at 100%. The separated peptides were analysed using a Thermo Scientific Q-Exactive mass spectrometer. Tandem mass spectra were collected in data-dependent acquisition (DDA) mode using a Top10 method.

2.6. Protein analysis and label-free data analysis

The raw data were processed in the MaxQuant software v1.6.0.16 using label-free intensity based absolute quantification (iBAQ). MS/MS data were searched against the custom in-house databases of predicted protein sequences encoded by *S. haemolyticus* 51-08 (ERS066281, 2527 coding DNA sequences) and *S. haemolyticus* 57-1 (genome assembly not published, 2518 coding DNA sequences). The false discovery rate (FDR) was controlled using a target-decoy approach and limited to 1%. The quantitative comparison of proteins in the two different bacterial

strains was performed using the relative iBAQ values (riBAQ) in Perseus programme v1.5.6.0 [17], filtered for proteins with minimum two peptides identified. All contaminants were first filtered out and the relative iBAQ values for each sample were log10 transformed. The ratios of proteins enriched in vesicles were then calculated using non-transformed riBAQ values. Data were then analysed for statistically significant changes using *t*-test. Only proteins identified in at least two replicates in at least one of the two groups were considered. Missing values were replaced from normal distribution using width = 0.3 and downshift = 1.8 settings. Differentially expressed proteins were then visualized using Volcano plot with FDR < 0.05 and artificial within group variance s0 = 0.3. For qualitative comparisons, only proteins present in at least two replicates in each group were considered further.

2.7. Bioinformatic analyses

The cellular localisation of each protein was predicted using the PSORTb subcellular localisation tool version 3.0.2 [18]. The presence of potential signal sequences in each peptide was identified using SignalP v4.1 [19,20]. Secretome P v2.0 was used to predict nonclassical protein secretion [21]. Functional annotation and grouping of proteins into orthologous groups were performed using EggNOG version 4.5.1 [22]. Proteins classified as hypothetical proteins by EggNOG were further analysed by using NCBI Conserved Domains [23]. Venn diagrams were made using the online tool Venny v2.1.0 [24].

3. Results

3.1. *S. haemolyticus* releases membrane vesicles

In order to evaluate whether *S. haemolyticus* produces MVs, AFM analysis of bacteria grown on agar plates were performed. The commensal strain of *S. haemolyticus* is surrounded by blebs that appear as vesicles (Fig. 1A) as previously demonstrated by AFM images of MV blebbing in *S. aureus* [13]. To study this further, MVs were isolated from a commensal and a clinical strain and purified further by gradient centrifugation, to remove contaminating proteins and cellular debris. The various fractions in the gradient were evaluated for protein content by SDS-PAGE see Fig. 1A in [25] and for presence of MVs by TEM analysis. TEM analysis showed that MVs appeared in fractions 2–7, and these fractions were therefore pooled. The size of *S. haemolyticus* MVs were evaluated by TEM, and ranged from 20 to 180 nm (Fig. 1B).

3.2. Characterisation of MV- associated proteins in clinical and commensal strain

We wanted to analyse and compare the proteins associated with the MVs from the commensal and clinical strain of *S. haemolyticus*. MVs purified by density gradient were precipitated, and the proteins associated with MVs identified by mass spectrometry. Based on the detection of the protein in at least two of the three parallel experiments, 313 and 440 see Table 1 in [25] proteins were identified in the MV fractions of the clinical and the commensal strain respectively. The two strains shared 268 MV associated proteins (Fig. 2A), while the clinical and commensal strain had 73 and 206 unique proteins, respectively, see Table 2 in [25].

The proteins were further grouped into orthologous groups by EggNOG. The overall protein distribution was similar in the two strains, with a high proportion of proteins with unknown function, and proteins involved in translation, ribosomal structure and biogenesis and amino acid transport and metabolism (Fig. 2B). Several lipoproteins involved in iron and cation acquisition and transport were found in the MV cargo of both strains. In both strains we identified the surface protein SasC and two proteins with a LysM motif, allowing non covalent attachment to the cell wall, in addition to several cytoplasmic proteins with known moonlighting functions see Table 2 in [25].

Proteins involved in intracellular trafficking and vesicular transport were found only in the MV cargo of the clinical strain. Proteins only found in the MVs of the commensal strain were involved in Cell cycle control, cell division, chromosome partitioning; nucleotide transport and metabolism; post-translational modification, protein turnover and chaperones and signal transduction mechanisms. Moreover, the MV cargo of the clinical strain was associated with more proteins involved in defence mechanisms, replication, recombination and repair, translation, ribosomal structure and biogenesis, transcription, cell wall/membrane/envelope biogenesis and proteins of unknown function, compared to the low virulent commensal strain (Fig. 2B). The commensal isolate had more proteins involved in energy production and conversion, inorganic ion transport and metabolism, and amino acid transport compared to the clinical strain (Fig. 2B).

Among the unique MV associated proteins of the clinical strain, proteins involved in antimicrobial resistance were found. Beta lactamase class A and D, hydrolysing the ring of beta lactam antibiotics, the penicillin binding protein mecA and AACA-APH conferring resistance to aminoglycosides see Table 2 in [25]. The beta lactamase as well as two

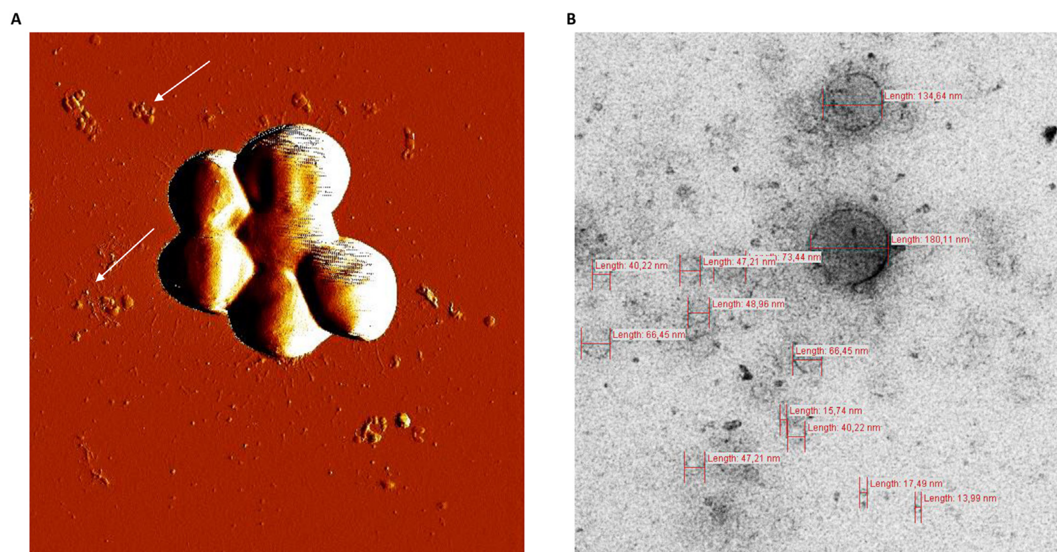

Fig. 1. *S. haemolyticus* releases membrane vesicles in vitro. A: Atomic force microscopy images of MVs from an *S. haemolyticus* clinical strain grown on LB agar plates. Arrows indicate surrounding membrane vesicles. B: TEM of purified MVs from the commensal *S. haemolyticus* strain.

Table 1
The ten most abundant MV associated proteins uniquely identified in the *S.haemolyticus* strains. Proteins written in bold are genes and proteins of particular interest due to their virulence or antimicrobial resistance properties.

Gene identifier	NCBI accession	Gene name	Protein description	Major function
***S. haemolyticus* clinical strain**				
7067_4_39_Contig_79_gene_1	WP_103416081.1	*NA*	**Collagen binding protein**	Adhesion
7067_4_34_Contig_11_gene_24	WP_279808	*SH2354*	Major capsid protein	Phage related
7067_4_34_Contig_11_gene_53	WP_279808	*SH1792*	Single-strandedDNA-binding protein	DNA binding
7067_4_39_Contig_11_gene_25	WP_053019334	*SH0936*	Hydroxyacyl-[acyl-carrier-protein] dehydratase	Lipid synthesis
7067_4_39_Contig_57_gene_4	WP_279808	*SH1764*	**Beta-lactamase**	Response to antibiotic
7067_4_39_Contig_14_gene_39	WP_033079618	*SH0827*	30S Ribosomal protein S10	Translation
7067_4_34_Contig_67_gene_1	WP_279808	*SH2426*	**YSIRKsignal domain/LPXTG**	Adhesion
7067_4_39_Contig_11_gene_5	WP_279808	*SH2355*	Uncharacterised protein	Viral scaffold
7067_4_39_Contig_8_gene_66	WP_087503638	*SH0181*	3-methyl-2-oxobutanoate hydroxymethyltransferase	Coenzyme transport
7067_4_39_Contig_11_gene_54	WP_049426210	*AL487_004505*	Single-strandedDNA-binding protein	DNA binding
7067_4_39_Contig_29_gene_28	WP_279808	*SH1546*	Tautomerase	Isomerase
***S. haemolyticus* commensal strain**				
SH_1_Contig_14_gene _74	WP_011275085.1	*CHAP-domain containing protein*	**Surface antigen**	NA
SH_1_Contig_1_gene _112	WP_053022007.1	*AL487_004355*	Phage capsid protein	Capsid protein
SH_1_Contig_1_gene _97	WP_049395108.1	*SH2368*	Trimeric dUTPase	dUTP diphosphatase activity
SH_1_Contig_1_gene _77	WP_06631851.1	*Q4L341*	Single strand binding protein	DNA binding
SH_1_Contig_1_gene _110	WP_033079738.1	*SH0576*	Gluconate permease	Transmembrane transport
SH_1_Contig_1_gene _111	WP_070499237.1	*SH2358*	Phage terminase	DNA packaging
SH_1_Contig_1_gene _106	WP_070487579.1	*B8W97_13505*	Phage holin	Phage related functions
SH_1_Contig_4_gene _25	WP_037550722	*DUF 1229*	DUF domain protein	NA
SH_1_Contig_1_gene _103	WP_107634554.1	*DUF722*	DUF domain protein	NA
SH_1_Contig_1_gene _54	WP_053018964.1	*SH1816*	Thioredoxin	Cell redox homeostasis

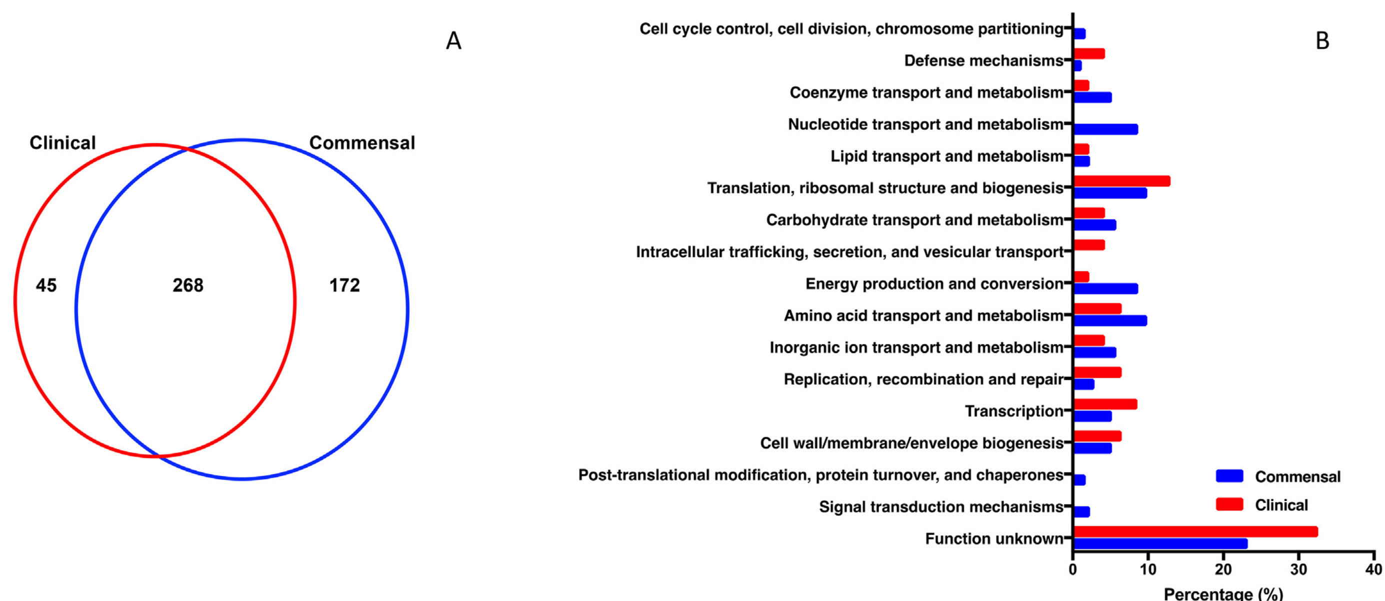

Fig. 2. Functional classification of proteins identified in membrane vesicles. A: VENN diagram showing number of shared and unique proteins associated with membrane vesicles from the clinical and the commensal strain. B: EggNOG based classification of proteins (%) associated with the MVs of the two strains.

proteins with an LPXTG motif known to mediate covalent attachment to the cell wall in adhesive proteins were among the top 10 most abundant proteins in vesicles from this strain (Table 1).

Of particular interest are several proteins involved in uptake of iron found in the MVs from the commensal strain see Table 2 in [25]. Two proteins with an YSIRK/LPXTG motif, were also identified among the most abundant proteins in MVs of the commensal strain (Table 1). The EggNOG analysis classified several proteins with unknown function (Fig. 2B). Manual BLAST and Conserved domain searches of the proteins with unknown function identified them as originating from bacteriophages, and as proteins containing LPXTG, YSIRK motifs, which are abundantly found in the vesicles see Table 2 in [25].

The sub-cellular localisation and origin of the MV associated proteins from the two strains were analysed by the PSORTB software. The majority of the identified MV associated proteins in both strains were classified as cytoplasmic proteins (50% and 71% from the clinical and commensal strain respectively), as shown in Fig. 3. The clinical strain had higher proportion of extracellular as well as cell wall proteins and proteins with un-classified localization, compared to the commensal strain (Fig. 3). In contrast, the commensal had a higher proportion of cytoplasmic proteins associated with membrane vesicles compared to the clinical strain (Fig. 3).

The identified MV associated proteins were further analysed by SignalP and SecretomeP. SignalP predicts presence and location of signal-peptide cleavage sites in the protein sequences, while SecretomeP predicts non-classical protein secretion. Signal P predicted that 7.0% and 4.6% of the clinical and commensal MVs associated proteins had a signal peptide, respectively. Similarly, the analysis with

Table 2
The ten most abundant proteins uniquely identified in the Total Secretome of the *S.haemolyticus* strains. Proteins written in bold are of particular interest due to their virulence or antimicrobial resistance properties.

Gene identifier	NCBI accession	Gene name	Protein description	Major function
***S. haemolyticus* clinical strain**				
7067_4_39_Contig_79_gene_1	WP_103416081.1	*NA*	**Collagen binding protein**	Adhesion
7067_4_39_ Contig_4_gene_35	WP_011274688.1	*NA*	Hypothetical protein	NA
7067_4_39_Contig_41_gene_16	WP_037558933.1	*NA*	Hypothetical protein	NA
7067_4_39_Contig_41_gene_3	WP_016930889	*NA*	Hypothetical protein	NA
7067_4_39_Contig_67_gene_1	WP_01127668.1	*NA*	**YSIRKsignal domain/LPXTG anchor domain protein**	Adhesion
7067_4_39_Contig_4_gene_29	WP_053029024.1	*NA*	**LPXTG anchor protein**	Adhesion
7067_4_39_Contig_18_gene_52	WP_053019334.1	*SH0936*	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	Fatty acis biosynthesis
7067_4_39_ Contig_4_37	WP_016930626.1	*NA*	Hypothetical protein	NA
7067_4_39_Contig_11_gene_53	WP_053024457.1	*NA*	HU family DNA binding protein	DNA binding protein
7067_4_39_Contig_79_gene_1	WP_053017623	*NA*	LysM, **CHAP domain protein**	Adhesion
***S. haemolyticus* commensal strain**				
SH_Contig_1_gene_112	WP_053022007.1	*NA*	Phage capsid protein	NA
SH_Contig_14_gene_3	WP_011275150.1	*SH0836*	30S ribosomal protein S9	Translation
SH_Contig_1_gene_97	WP_049395108.1	*SH 2368*	dUTP phospahatase	Polypeptide binding
SH_Contig_14_gene_30	WP_107641137.1	*SH0805*	50S ribosomal protein L22	Translation
SH_Contig_1_gene_77	WP_066031851.1	*SH0983*	Single strand DNA binding protein	Dna binding
SH_Contig_16_gene_9	WP_053019334.1	*SH0936*	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	Fatty acid biosynthesis
SH_Contig_20_gene_8	WP_053018515	*SH2467*	50S ribosomal protein L7	Polypeptide binding
SH_Contig_1_gene_111	WP_107610065	*SH1858*	Pyruvate dehydrogenase	Catalytic activity
SH_Contig_5_gene_111	WP_053027994.1	*B8W97_11210*	D-alanyl-D-alanine carboxypeptidase	Peptidase
SH_Contig_3_gene_51	WP_016930747.1	*NA*	Hypothetical protein	NA

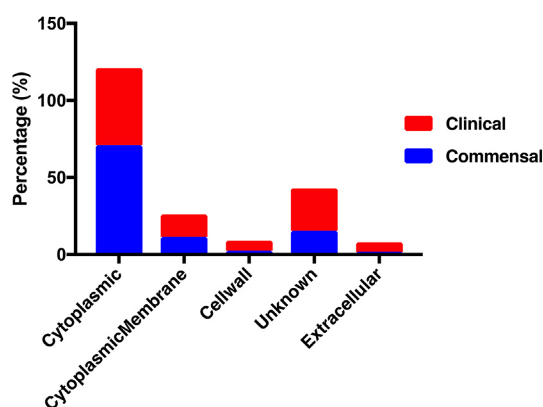

Fig. 3. Predicted subcellular origin using Psortb, of the MV associated proteins isolated from the clinical and the commensal strain.

SecretomeP predicted that 2.2% and 9.9% of the MV-associated proteins in the clinical and commensal strain respectively, were non-classically secreted proteins.

3.3. Characterisation of total secretome

We also wanted to compare the total secretome (TS) of the two *S. haemolyticus* strains. The bacterial culture filtrates were precipitated and the proteins were analysed by MS. In total 241 and 493 proteins were identified in the supernatant of the clinical isolate and the commensal isolate respectively, and among them 199 proteins were shared see Tables 1 and 3 in [25].

The distribution of clusters of orthologous groups (COG) in the total secretome from the two isolates was overall similar. The majority of proteins belonged to the COG groups translation, ribosomal structure and biogenesis, amino acid transport and metabolism, energy production and conversion, and function unknown (Fig. 4). When comparing the COG distribution of total secretome between the two strains, the clinical strain had more proteins classified as involved in defence mechanisms, cell wall/membrane/envelope biogenesis and function unknown compared to the commensal strain. In contrast, the commensal strain had more proteins classified as proteins involved in nucleotide transport and metabolism, coenzyme transport and metabolism, translation, ribosomal structure and biogenesis and energy production and conversion.

In order to further determine if the strain specific protein secretome was due to the strain specific response to the growth conditions, functional classification was performed for the total proteome encoded in the genomes of the two strains, and further compared to the strain specific secreted proteins. The proteomes, predicted from the genome sequences of the two strains are very similar. The observed differences in secreted proteins, as shown in Fig. 4, might be due to a differential gene expression of the two strains.

The most abundant proteins identified in the secretome of both strains were Phenol soluble modulin (PSM) beta 1–3 and PSM α see Table 3 in [25]. The cytoplasmic proteins glyceraldehyde −3-phosphate- isomerase (GAPHD), enolase, aldolase, glucose-6-Phosphate isomerase, Inosine 5` monophosphate, GroEL, triose-phosphate-isomerase and glutamine synthetase were found in the TS of both strains. These proteins are known to have dual function and are denoted as moonlighting proteins.

Moreover, the clinical isolate had 42 strain specific proteins while the commensal isolate had 294 strain specific proteins in the TS. Proteins associated with antimicrobial resistance (penicillin binding proteins) and proteins with LPXTG/YSIRK motifs were detected in the TS of the clinical strain, among the top ten most abundant proteins three proteins with adhesive properties were found (Table 2).

The commensal strain had more proteins in the TS associated with acquisition of iron compared to the clinical isolate, among the top ten most abundant proteins were several ribosomal proteins, and the moonlighting protein Pyruvate dehydrogenase (Table 2).

3.4. *S. haemolyticus* MVs are enriched in certain proteins compared to proteins detected in the total secretome

In order to determine if the *S. haemolyticus* MVs are indeed enriched in virulence factors, we compared the MV cargo to the total secretome. If proteins were detected with a threshold detection rate of FDR 0.05 in the MV sample as compared to in the TS, these proteins were defined as enriched.

The MV samples of the clinical and commensal strain were enriched in 98 and 131 proteins compared to the TS see Table 4 and Figs. 1 and 2

Table 3
The 20 most abundantly enriched proteins identified in the membrane vesicles compared to the total Secretome of *S.haemolyticus*. Genes and proteins written in bold are of particular interest due to their virulence or antimicrobial resistance properties.

Gene identifier	NCBI accession	riBAQ MV x/ y_riBAQ TS	Gene name		Protein description
***S. haemolyticus* clinical strain**				279808.SH2009	
7067_4_39_Contig_15_gene_42	WP_011276276.1	36,5	*PEPA*	279808.SH0805	Aminopeptidase
7067_4_39_Contig_14_gene_43	WP_107641137.1	33,8	*rplB*	396513.Sca_0311	50S ribosomal protein L2
7067_4_39_Contig_21_gene_2	WP_015899569.1	33,6*	***LysM***	279808.SH1764	**lysM**
7067_4_39_Contig_57_gene_4	WP_279808.1	31,3	***BLAZ***	279808.SH1060	**Beta-lactamase**
7067_4_39_Contig_6_gene_86	WP_107612164.1	29,3	***FTNA***	279808.SH1002	**Bacterial non-heme ferritin**
7067_4_39_Contig_6_gene_32	WP_011275310.1	22,5	*groEL*	Q3L881	Chaperone
7067_4_39_Contig_14_gene_63	WP_053041491.1	22	*rplM*	2789808.SH2354	50 SRibosomal protein L13
7067_4_39_Contig_11_gene_24	WP_279808.1	20,5		279808.SH1856	Major capsid protein
7067_4_39_Contig_28_gene_23	WT_053024633.1	20,35	*PDHD*	2789808.SH2468	Dyhydrolipoyl dehydrogenase
7067_4_39_Contig_31_gene_20	WP_085060703.1	19,9	*rplJ*	2789808.SH1273	50S ribosomal protein L10
7067_4_39_Contig_10_gene_20	WP_066031883.1	17,3	*rplU*	2789808.SH1471	SH 1273, rplu,50s ribosomal protein
7067_4_39_Contig_87_gene_1	WP_011275766.1	14,7		2789808.SH2250	YSIRK containing signal peptide
7067_4_39_Contig_16_gene_23	WP_011276510.1	15,3	*tagF*	2789808.SH0836	Glycosyltransferase, group 2 family protein
7067_4_39_Contig_14_gene_72	WP_011275150	13,9	*rpsI*	2789808.SH0907	Ribosomal protein S9
7067_4_39_Contig_18_gene_23	WP_053019918.1	12,5	*rpoE*	2789808.SH2583	Probable DNA-directed RNA polymerase subunit delta
7067_4_39_Contig_21_gene_31	WP_053021514.1	11,2	*guaB*	2789808.SH1018	Inosine-5-monophosphate dehydrogenase
7067_4_39_Contig_6_gene_45	WP_104948354.1	9,76	*bamJ*	279808.SH0863	Aminotransferase
7067_4_39_Contig_38_gene_5	WP_011275175.1	9,1	***FECB***	279808.SH1975	**Iron(III) dicitrate-binding protein**
7067_4_39_Contig_15_gene_9	WP_011276242.1	9	*clpB*	1005058.UMN179_01656	Chaperone protein ClpB
7067_4_39_Contig_13_gene_27	WP_048667800	8,5	*ALDA*	Q4L919	Aldehyde dehydrogenase
***S.haemolyticus* commensal strain**					
SH_1_Contig_30_gene_13	WP_011275438.1	144,6	*PCKA*	279808.SH1137	Phosphoenolpyruvate Carboxylase
SH_1_Contig_2_gene_35	WP_053041297.1	56,1	*GBSA*	B8W97_04095	Betaine- Aldehyde Dehydrogenase
SH_1_Contig_17_gene_15	WP_053041210.1	40,7	*NA*	B8W97_11220	Poly alpha -glucosyltransferase
SH_1_Contig_4_gene_143	WP_037569827.1	40	*PEPA*	279808.SH1177	Peptidase m42 family protein
SH_1_Contig_20_gene_16	WP_053023376.1	36,2	***SASH***	279808.SH2452	**LPXTG surface 5'-nucleotidase**
SH_1_Contig_2_gene_88	WP_053019568.1	35,9	*SRAP*	279808.SH0326	**Serine-rich glycoprotein adhesin SraP family protein**
SH_1_Contig_1_gene_333	WP_053017719.1	32,9	*YHFE*	279808.SH1543	Metallo hydrolase
SH_1_Contig_3_gene_134	WP_048667800.1	32,6	*ALDA*	279808.SH0547	Aldehyde dehydrogenase
SH_1_Contig_21_gene_2	WP_011275466.1	30,3	***SASC***	279808.SH1165	**YSIRK/LPXTG surface protein,**
SH_1_Contig_11_gene_15	WP_053027580.1	26,8	*SUCA*	279808.SH1492	2-oxoglutarate dehydrogenase, E1
SH_1_Contig_22_gene_9	WP_011276736.1	23,9	*CLPC*	279808.SH2484	ATP-dependent Clp protease
SH_1_Contig_15_gene_22	WP_011276260.1	23,21	*ROCD*	279808.SH1993	Ornithine aminotransferase
SH_1_Contig_1_gene_246	WP_053024043	22,9	*YMFH*	279808.SH1634	Peptidase, M16
SH_1_Contig_3_gene_172	WP_011274837.1	22,9	*NA*	435838.HMPREF0786_01053	CHAP domain protein, Surface antigen
SH_1_Contig_1_gene_256	WP_053028931.1	22,6	*PORA*	279808.SH1624	Oxidoreductase
SH_1_Contig_8_gene_49	WP_011276401.1	22,3	*RAIA*	279808.SH2139	Ribosomal subunit Interface protein
SH_1_Contig_1_gene_76	BAE05102.1	20,36	*STERM_0814*	279808.SH1793	Erf family
SH_1_Contig_3_gene_164	WP_033079754.1	17,8	*GLCB*	279808.SH0358	The phosphoenolpyruvate-dependent sugar phosphotransferase system
SH_1_Contig_17_gene_14	WP_048667926.1	16,3	*SGAA*	279808.SH1201	Aminotransferase, class V
SH_1_Contig_4_gene_120	OJH00147.1	16,33	*NA*	272620.KPN_00994	**YSIRK domain surface protein**

in [25]. The MVs of both the clinical and the commensal strain were enriched in LPXTG and LysM motif containing surface proteins, lipoproteins and proteins involved in uptake of iron.

The MVs from the clinical strain were also enriched in proteins involved in antimicrobial resistance, beta lactamase blaZ, and AACA-APH conferring resistance to beta lactams and aminoglycosides and the moonlighting proteins aldolase and Inosine-6-P-isomerase see Table 4 in [25]. Among the top 20 most enriched proteins in the clinical strain (Table 3), Lys M was found at a 24.4 times higher ratio in the MVs compared to in the TS. Lys M is a protein known to confer cell wall attachment to several surface proteins [26], and also confers adhesion to fibrinogen and fibronectin [27]. Ferritin and iron (III) binding proteins were found at 29.3 and 9.1 higher ratios in the MVs compared to the TS while BlaZ was 29.1 times more abundantly found in the MVs compared to the TS in the clinical strain.

In the commensal strain proteins with an LPXTG/YSIRK motif were also among the 20 most abundantly enriched proteins in the MVs. The surface proteins SraP and sasC were found 35.9 and 30.3 times more abundantly in the MV compared to the TS, additionally two LPXTG motif proteins were found at ratios 16 and 36 times more abundant in the MVs compared to the TS (Table 3).

The moonlighting proteins GAPHD, Enolase and Pyruvate dehydrogenase were also enriched in MVs of the commensal strain see Table 4 in [25].

4. Discussion

Secretion of outer membrane vesicles by Gram-negative bacteria has been known for decades and thoroughly described [28]. Several publications have demonstrated the importance of membrane vesicles in bacterial communication, immune evasion, protection against antimicrobial agents and implications in bacterial virulence [29–31]. The production of membrane vesicles from Gram-positive bacteria was first described in *S. aureus* by Lee et al. in 2009 and is now described in several Gram positive bacteria [29,32,33]. In a study by Siljamaki et al. 2014, non-classical protein secretion in MVs was suggested for *Staphylococcus epidermidis* [34].

4.1. Membrane vesicle content

S. haemoyticus is a part of the commensal flora and has few virulence factors, compared to *S. aureus*. However, *S. haemolyticus* has over the past decades emerged as an opportunistic nosocomial pathogen. We were interested to know if the membrane vesicle cargo differed in a

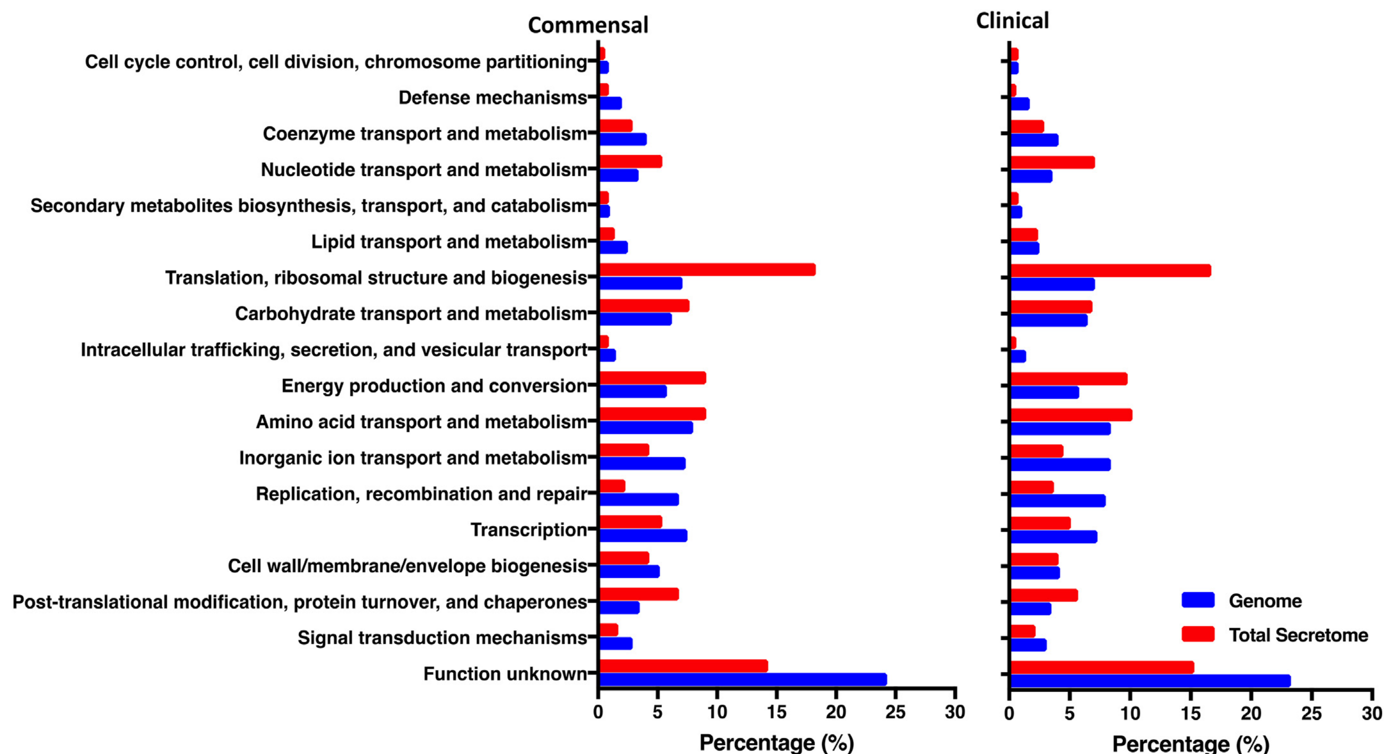

Fig. 4. EggNOG based classification of secreted proteins and proteome (based on genomes) of the two strains.

commensal and a clinical strain of *S. haemolyticus*, and if we could detect more proteins involved in virulence in the clinical strain compared to the commensal strain.

Differences both in the number of proteins as well as in the protein content were found.

The clinical strain had more proteins involved in antimicrobial resistance such as MecA and BlaZ mediating antimicrobial resistance to penicillin and methicillin and the bifunctional Aac-AphD which mediates antimicrobial resistance to gentamicin [14]. MVs have previously been shown to carry functional antimicrobial resistance genes [35], and in a proteomic analysis of the *S. aureus* and *S. epidermidis* MV cargo, the presence of active beta lactamases was demonstrated [32,34,35].

Adhesion is one of the main virulence mechanisms of CoNS, as it is the key step in colonization and infection [36]. YSIRK/LPXTG and LysM motifs are characteristic for adhesive proteins, and proteins with an LPTG/YSIRK/LysM motifs were enriched in the MV cargo of both strains. Adhesion proteins have been found in the MVs of *Pseudomonas putida*, *Bacillus subtilis* and in *Escherichia coli* where it was shown to increase adherence to host cells [30,37,38]. Release of MVs has been shown to increase during biofilm formation, indicating that they play an important role of delivery of DNA and proteins to shape the biofilm matrix [39,40]. Additionally MVs can act as a transport vehicle transporting virulence factors and effector proteins in a protected manner [41] mediating bacteria-bacteria interactions or bacteria –host interactions [10].

In agreement with previous studies [8,34,42], we found an enrichment of proteins involved in acquisition of iron in the MV cargo from both strains. Free iron is a limiting factor for bacterial growth, and in order to successfully proliferate on the human host several systems have evolved to sequester iron [43,44]. Prados-Rosalez et al. demonstrated that *Mycobacterium tuberculosis* produces MV packed with iron loaded mycobactin, a protein able to transfer iron from chelators, and deliver iron to adjacent bacteria under growth in iron limiting conditions [44]. Recently Lin et al. demonstrated that iron sequestering proteins are released in outer membrane vesicles of *Pseudomonas aeruginosa* under iron depleted growth circumstances [45].

The growth media used in this study is considered as a rich media, however, the strains were grown to late exponential phase/early stationary phase when less iron was probably available due to the consumption during growth. This might have induced the production of proteins involved in iron uptake. Interestingly the highest number of proteins involved in iron acquisition was found in MVs of the skin commensal. The iron acquisition proteins are membrane anchored lipoproteins, and has been shown to confer virulence and TLR2 activation in *S.aureus* [46]. Supply of free iron is a limiting factor for bacterial growth, and both the clinical and the commensal strain should be able to efficiently acquire iron in order to survive. Considering the different environment encountered in the blood and on the skin, one can speculate that MVs are a more efficient strategy for iron acquisition on the human skin, than in blood, resulting in the higher number of proteins involved in iron acquisition in the commensal strain.

4.2. MVs are enriched in specific proteins

In order to determine if the *S. haemolyticus* MVs are enriched in virulence factors, the strain specific MV cargo was compared to the strain specific total secretome. An enrichment analysis of the MV proteins showed that MVs in both strains were enriched in proteins involved in iron acquisition, antimicrobial resistance and adhesion. The identification of proteins involved in antimicrobial resistance in the MVS of the clinical *S. haemolyticus* strain, reflects the highly antibiotic resistant genotype and such pathogenesis –associated proteins have also been shown in *S. aureus* MVs [47].

4.3. The most abundant proteins in the total secretome were phenol soluble modulins

Interestingly the most abundant proteins in the total secretome in both strains were the phenol soluble modulins β 1–3 and PSM α. It was recently demonstrated that *S. haemolyticus*, in addition to PSM β 1–3, produces a novel PSM α type, with pronounced cytolytic activity [6]. PSMs are produced by *S. aureus* and most CoNS, and are important

mediators of virulence as cytolysins and attractants of neutrophils, cytokine production and lysis of erythrocytes [48,49]. Moreover, it was previously demonstrated that PSMs play an important role in staphylococcal biofilm development and colonization [50]. In addition, the strong surfactant property of PSMs can interact with the hydrophobic compounds secreted from sebaceous glands on the skin, which might enhance skin colonization by *S. aureus* [49]. It has previously been showed that PSMs are produced in large quantities, being the most abundant proteins in *S. epidermidis* culture supernatant [49].The widespread production of PSMs in the less virulent commensal CoNS species indicates that PSMs may play important roles in host colonization. This is in agreement with our results, where high levels of PSM β 1–3 and PSM α secretion was observed in both the commensal and in the clinical strain.

4.4. Moonlighting proteins

A large proportion of the proteins in the total secretome and MVs from both strains was of cytoplasmic origin, and the majority of the proteins had no known motifs for surface export. The identification of cytoplasmic proteins in the secretome and in the MVs can be explained by cell lysis. However it has been shown that several cytoplasmic proteins can function as moonlighting proteins, and that they are not excreted due to cell lysis [51]. Moonlighting proteins often have dual functions. While being engaged in cellular functions intracellularly they often have important adhesive functions extracellularly [52]. Several of the proteins found in both strains have previously been shown to have moonlighting functions, such as ribosomal proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPHD) and enolase [52,53]. GAPHD, fructose 1,6-bisphosphate aldolase and enolase were found in the TS and in the MVs of both the commensal and the clinical strain and are some of the best characterized moonlighting proteins. Studies have shown that they are involved in adhesion to several serum proteins and epithelial cells [53], which are important environmental constituents for both the commensal and clinical *S. haemolyticus* isolate.

5. Conclusion

We show that both clinical and commensal *S. haemolyticus* isolates produce membrane vesicles. Strain specific differences in secreted proteins, as well as MV cargo were found. Proteins such as BlaZ, mecA and Aac-AphD conferring antimicrobial resistance in addition to enriched proteins involved in adhesion was more abundantly found in the MVs of the clinical strain, reflecting it's more virulent traits. The MVs of both strains were enriched in proteins involved in acquisition of iron, but the MVs of the commensal strain contained more of these proteins.

Thus, identification of staphylococcal membrane vesicles can potentially be used in novel approaches to combat staphylococcal infections, such as development of vaccines.

Conflict of interest

None of the authors declares any conflict of interests.

Data availability

The mass spectrometry proteomics data have been deposited to the PRIDE database [54] via the PRIDE partner repository with the dataset identifier PXD010389. Proteins found in the totalsecretome and MVs are reported in detail in [25].

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