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Identifying *Staphylococcus haemolyticus* surface proteins

Development of a novel method for detection of bacterial surface proteins expressed during colonisation of human keratinocytes

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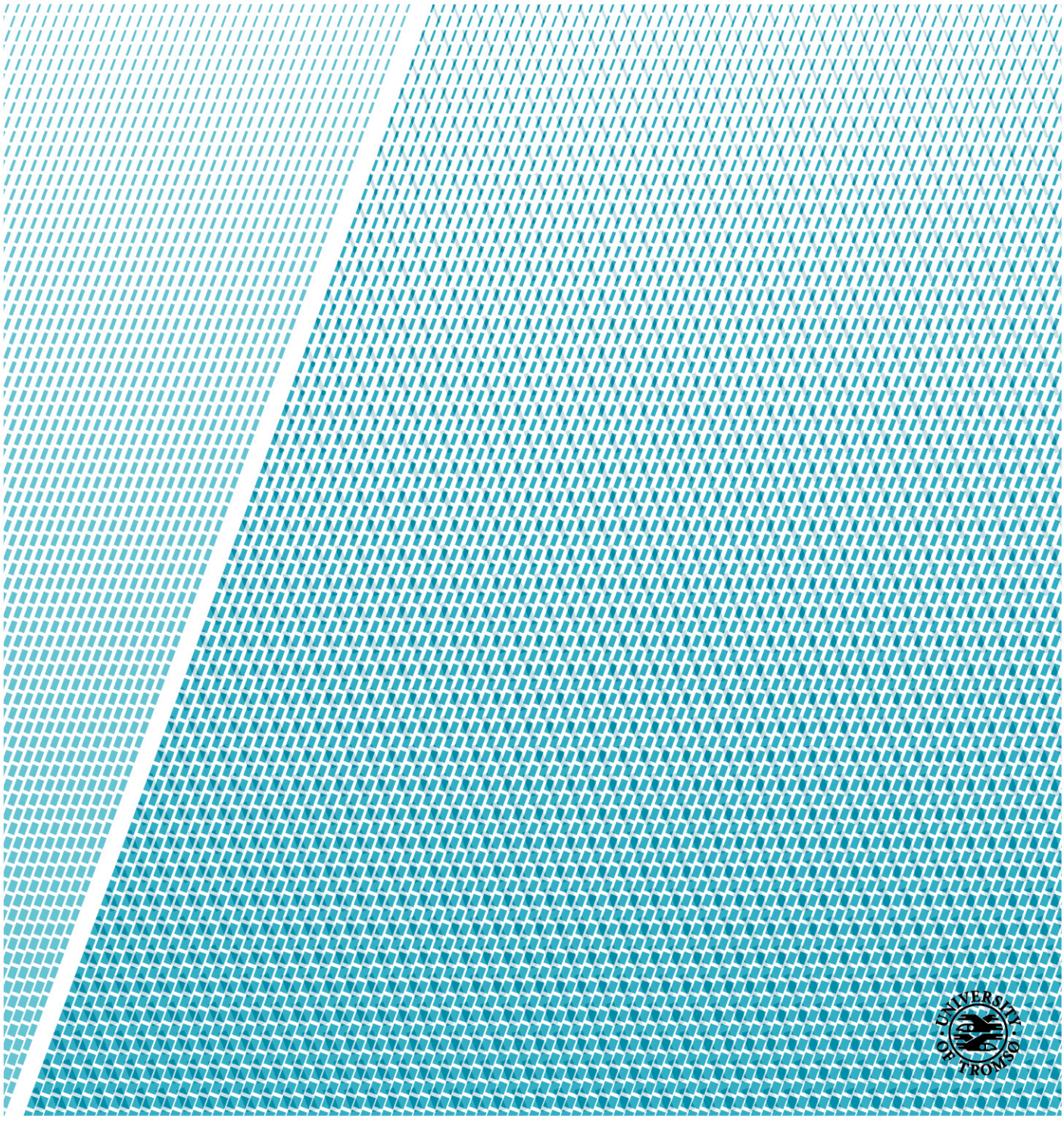

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Abstract

This assignment is based on the work done in MBI-3911 “Master thesis” to qualify for a Master of Science in biomedicine at UiT The Arctic University of Norway. The aim of this thesis was to develop a method for identification of expressed surface proteins of *Staphylococcus haemolyticus* when colonising human keratinocytes (HaCaT).

S. haemolyticus and HaCaT cells were grown together and separated with Fluorescence-activated cell sorting (FACS) before using the Lipid-based Protein Immobilization (LPI™) Flow Cell technology (Nanoxis Consulting AB) to do surface protein shaving of the bacteria. Tandem mass tags (TMT) were used to investigate the up- and downregulation of proteins comparing *S. haemolyticus* incubated and not incubated with HaCaT cells prior to surface shaving.

Surface shaving of *S. haemolyticus* was done twice; in an initial and in an optimized experiment. Optimizations of the method increased both the number of bacteria adhering to HaCaT cells and retrieval of bacteria after FACS. The most important change between the initial and the optimized experiment were the centrifugations. A centrifugation step was implemented when incubating bacteria and human cells, the centrifugation of overnight cultures were changed to a swing bucket centrifuge, and two ultracentrifugation steps were implemented prior to the LPI™ Flow Cell.

Three hundred and nineteen proteins were identified by MS in total: 18 strongly upregulated (fold change 1.53-3.82), 41 slightly upregulated (fold change 1.20-1.49), 62 slightly downregulated (fold change 0.67-0.83) and 14 strongly downregulated (fold change 0.49-0.66). Six adhesion and/or virulence proteins were detected among the 18 strongly upregulated proteins: three proteins with YSIRK/LPXTG motifs, one reported important for attachment to nares of rats, one AtlE protein and one heme oxygenase. Twelve cell wall and eight extracellular proteins were identified among the 319 proteins. 66% of the proteins were predicted to be from cytoplasmic origin.

An alternative approach where surface shaving of *S. haemolyticus* in overnight cultures of used and unused cell culture medium was also performed, and 794 proteins were found. The up- and downregulation of proteins in the alternative approach were not

directly comparable to the up- and downregulation of proteins in the HaCaT colonisation model, even though some of the detected proteins were similar. A higher number of proteins were found using the alternative surface shaving approach, and a higher rate of predicted cytoplasmic proteins, possibly indicating a higher degree of bacterial lysis.

1 Abbreviations and explanations

AMP	antimicrobial peptide
Atl	autolysin
bp	base pair
BSI	blood stream infection
CAPD	continuous ambulatory peritoneal dialysis
CDS	coding DNA sequence
CFU	colony forming unit
CHAP	cysteine-histidine-dependent amidohydrolases/peptidases
COG	cluster of orthologous groups
CoNS	coagulase-negative staphylococci
CoPS	coagulase-positive staphylococci
CWA	cell wall anchored protein
DLL	dock, lock and latch mechanism in proteins
DMEM	dulbecco´s modified eagle´s medium
EPM	extracellular polymeric matrix
FACS	fluorescence-activated cell sorting
FBRI	foreign body related infection
FBS	fetal bovine serum
FSC	forward scatter
ICAT	isotope-coded affinity tags
iTRAQ	isobaric tags for relative and absolute quantitation
LC	liquid chromatography

LPI™	lipid-based protein immobilization technology
LPXTG	Leu-Pro-X-Thr-Gly; where X can be any amino acid; a characteristic C-terminal sequence motif in MSCRAMMs
LysM	lysin motif
MOI	multiplicity of infection
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSCRAMM	microbial surface component recognizing adhesive matrix molecules
NVE	native valve endocarditis
OD	optical density
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PIA	polysaccharide intercellular adhesin
SCOPE	surveillance and control of pathogens of epidemiological importance
SD	Standard deviation
Sdr / SD	serine-aspartate repeat
SILAC	stable isotope labelling with amino acids in cell culture
SSC	side scatter
SSR	serine repeat region
TMT	tandem mass tags
TLR	toll-like receptor
TSB	tryptic soy broth; a general-purpose broth medium for cultivation of microorganisms

2 Introduction

2.1 Aim and hypothesis

The aim of this thesis was to develop a method for identification of expressed surface proteins of *Staphylococcus haemolyticus* when colonising human keratinocytes.

Our hypothesis was that *S. haemolyticus* would express specific surface proteins important for adhesion to the host upon contact with human cells.

2.2 Background and approach

Bacteria must colonize their host to cause infections. Commensal and bacterial pathogens express adhesive molecules on their surface that promote attachment and interaction with eukaryotic host cell receptors (Kline et al., 2009). Adhesion is the first step prior to invasion and/or secretion of toxins (Letourneau et al., 2011).

S. haemolyticus is part of our normal flora as well as an important opportunistic pathogen in hospital-acquired (nosocomial) foreign body related infections (FBRIs), particularly affecting immunocompromised patients and premature new-borns (Becker et al., 2014, Cavanagh et al., 2014, Czekaj et al., 2015).

Improved knowledge of *S. haemolyticus* adhesins are important in order to better understand host-pathogen interactions for further use in the design of novel vaccines. As an approach to this we suggest a method combining digestion ("cell shaving") and identification of surface exposed proteins after bacterial-host-co-incubation. We designed our experiment to include a pre-incubation step where *S. haemolyticus* attached to human keratinocytes in order to make the experiment more biologically relevant than when doing shaving of proteins without prior bacterial/human cell contact.

Methods for studying bacterial adhesion to Mammalian cells or surface protein shaving of bacteria are described (Solis and Cordwell, 2016, Letourneau et al., 2011, Askarian et al., 2014, Hilleringmann et al., 2009, Olaya-Abril et al., 2014, Rodriguez-Ortega et al., 2006, Tjalsma et al., 2008), and a variation of the surface shaving technology has been

described where host proteins interact with bacterial surface proteins (Boleij et al., 2011, Dreisbach et al., 2011). However, none of the methods combine a bacterium/eukaryotic cell incubation step prior to surface shaving of bacterial proteins.

2.3 Human keratinocytes

The skin provides a protective physical barrier between the body and the surroundings (e.g. pathogenic microorganisms).

Cell types found in the epidermis include melanocytes, Langerhans cells, and Merkel cells. Keratinocytes are the major cell type, making up approximately 95% of the epidermal cells (Nestle et al., 2009, Khavkin and Ellis, 2011). When the keratinocyte progressively moves from the basement membrane towards the skin surface, it forms four morphologically different layers (strata); stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Nestle et al., 2009, Khavkin and Ellis, 2011).

Keratinocytes are able to sense pathogens and mediate immune responses. Receptors (e.g. Toll-like receptors (TLRs)) recognizing pathogen-associated molecular patterns (PAMPs) can lead to innate and adaptive immune responses (Nestle et al., 2009). Keratinocytes can produce antimicrobial peptides (AMPs), cytokines and chemokines, and thereby react to harmful pathogens (Nestle et al., 2009).

2.4 Staphylococci with focus on *S. haemolyticus*

All mammals are exposed to and colonized by microorganisms during and after birth. The microbiota associated with a healthy body is dependent on body site, and there is also variation in the microbiota between individuals (Grice and Segre, 2011, Human-Microbiome-Project-Consortium, 2012).

S. haemolyticus is part of the coagulase-negative Staphylococci (CoNS) group, which represent a part of the microbiota of the skin and mucous membranes of both humans and animals (Grice and Segre, 2011, Becker et al., 2014, Cavanagh et al., 2016). CoNS are opportunistic pathogens, meaning that they can cause disease in an immunocompromised host, and they are today one of the major nosocomial pathogens (Wisplinghoff et al., 2004, Urzedo et al., 2014, Becker et al., 2014).

The most described species among CoNS is *Staphylococcus epidermidis*. CoNS are also sometimes described as a whole group, not differentiating between the different species. Literature regarding *S. haemolyticus* can be found, mostly related to levels of antimicrobial resistance. In general the published information on virulence factors is scarce compared to literature published on other staphylococcal species.

2.4.1 Classification and characteristics

The nonsporulating Gram-positive cocci of the genus *Staphylococcus* is a part of the *Staphylococcaceae* family, *Bacillales* order, *Bacilli* class and *Firmicutes* phylum (Madigan, 2009). Staphylococci (Greek: *staphylē*, "grape") often stick together in grape-like clusters.

Staphylococci have traditionally been divided in two groups; coagulase-negative (CoNS) or coagulase-positive (CoPS), considering coagulase as a major virulence factor (Table 1) (Becker et al., 2014). A CoPS commonly associated with human disease is *Staphylococcus aureus*. *S. aureus* and some other staphylococci produce the enzyme coagulase associated with pathogenicity. Coagulase induces fibrin clotting, which will hide the bacteria from the host defence system and prevent phagocytosis (Madigan, 2009).

The first descriptions of staphylococci were done in the late 19th century. Since then 47 species and 23 subspecies of *Staphylococcus* are validly described, and of these 38 are categorized as coagulase-negative species (Becker et al., 2014).

Table 1 - Human-associated staphylococci considering coagulase as a major virulence factor. The table is a modified version of Fig. 2 from Becker et al. (Becker et al., 2014), where the S. epidermidis-like group, Staphylococcus lugdunensis and Staphylococcus saprophyticus belong to the medium-pathogenic group of CoNS – predominately colonizing indwelling catheters (Becker et al., 2014). Compared to other C0NS, S. saprophyticus is not that common as a cause of catheter induced infections (Hedman and Ringertz, 1991, Raz et al., 2005). The main reason for this is that S. saprophyticus primarily adhere to uroepithelial cells, and other CoNS predominately colonize indwelling catheters (Raz et al., 2005). S. lugdunensis is somewhat different than other CoNS. It does not cause infection at the same frequency as S. aureus or S. epidermidis, but the severity of infection can resemble those caused by S. aureus (Frank et al., 2008).

Staphylococcus genus						
Coagulase-negative staphylococci (CoNS)						**Coagulase-positive/variable staphylococci (CoPS)**
***S. epidermidis*-like species:** *S. epidermidis, S. haemolyticus, S. capitis, S. hominis, S. pettenkoferi, S. simulans, S. warneri* and others		*S. saprophyticus* subsp. *saprophyticus*		*S. lugdunensis*		*S. aureus* subsp. *aureus*

2.4.2 Natural habitat

Staphylococci constitute a large proportion of the microbiota of the skin and mucous membranes of humans and animals (Grice and Segre, 2011, Becker et al., 2014). Cavanagh *et al.* analysed the body site distribution of CoNS from 114 healthy adults. Six body sites were swabbed on each participant (both arm pits, both knee pits and both sides of the groin). *S. epidermidis* was the dominant species and *Staphylococcus hominis* the second most dominant species on all body sites (Cavanagh et al., 2016). *S. haemolyticus* was the third, fourth and fifth most dominant species in the groin, knee pits and arm pits, respectively (Cavanagh et al., 2016).

2.4.3 Clinical significance

Of staphylococci, the CoPS species *S. aureus* is most frequently associated with human disease. Skin and soft-tissue infections, bacteraemia, toxic shock syndrome, meningitis, endocarditis and foodborne intoxications are some examples of the illnesses *S. aureus* can cause (Tong et al., 2015, Todd, 1997). Even though *S. haemolyticus* and other CoNS do not have the large repertoire of virulence factors found in *S. aureus*, they can cause a variety of different infections. CoNS are important emerging, opportunistic, nosocomial pathogens, and the most common infections caused by CoNS are foreign body related infections (FBRIs) due to biofilm formation (Otto, 2004, Becker et al., 2014, von Eiff et al., 2005).

Some examples of foreign polymer implants are venous or arterial catheters, mechanical heart valves, orthopaedic devices or implants. Contamination of the medical devices can come from the skin or mucous membranes of the patients or the hands of the clinical staff (von Eiff et al., 2005). The FBRIs can be local, such as CONS peritoneal infections due to continuous ambulatory peritoneal dialysis (CAPD) (Becker et al., 2014), or systemic such as blood stream infection (BSI). BSIs occur through release of bacteria from the colonized infection foci. The U.S. nationwide Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) detected 24179 cases of nosocomial BSIs in 1995-2002. CoNS were the most common cause of BSIs with 31% of the isolates; *S. aureus* caused 20% and enterococci 9% of the BSIs (Wisplinghoff et al., 2004).

There is an increase in patient groups with an impaired immune system (e.g. premature babies, immunocompromised patients and elderly), which have higher risk of infections with opportunistic pathogens such as CoNS (Becker et al., 2014). CoNS can also cause infections not related to foreign bodies, such as native valve endocarditis (NVE) (Selton-Suty et al., 2012) or surgical site infections (Weiner et al., 2016).

Of all surgical site infections reported to Centers for Disease Control and Prevention's National Healthcare Safety Network in the USA in 2011-2014, 7.9% was caused by CoNS. Only *Escherichia coli* and *S. aureus* had higher frequencies, with 13.7 and 20.7% of the cases, respectively (Weiner et al., 2016).

2.4.4 Antimicrobial resistance

Usually there are differences in the prevalence of antimicrobial resistance depending on whether bacteria are collected from outside a hospital environment (community strains) or inside a hospital environment (nosocomial strains), where they have been exposed to an antibiotic selection pressure. Antimicrobial resistance genes may be acquired by horizontal gene transfer between closely related staphylococcal species, or between different species (Becker et al., 2014).

Nosocomial isolates of *S. haemolyticus* are commonly found to be clinically resistant to several antimicrobial agents. One thousand two hundred and fourteen CoNS were collected from bacteraemia episodes in 25 hospitals in UK and Ireland in 2001-2006. *S. haemolyticus* was more resistant than the other species, with 84% of the isolates non-

susceptible to three or more classes of antimicrobial agents, compared to 70% of the *S. epidermidis* isolates. The prevalence of methicillin-resistant CoNS ranged from 54.2-79.9%, and was strongly correlated with multi-resistance (Hope et al., 2008). In a collection of 134 clinical European *S. haemolyticus* isolates, multi-resistance for a majority of the strains were reported (Cavanagh et al., 2014).

Antibiotic susceptibility testing on 386 CoNS community isolates from healthy adults in Norway revealed that the community isolates had a much lower prevalence of antimicrobial resistance than reported in nosocomial CoNS isolates. 5.2% of the isolates were resistant to three or more classes of antimicrobial agents and 4.1% of the CoNS isolates were methicillin-resistant (Cavanagh et al., 2016). A Portuguese study on community CoNS reported overall higher prevalence of resistance than in the Norwegian isolates (Oliveira and Cerca, 2013).

2.4.5 Virulence and biofilm formation

Virulence is the ability of a pathogen to cause disease (Madigan, 2009). Different virulence factors make it easier for the bacteria to invade the host, cause disease, and evade host defences (Peterson, 1996). One of the most important virulence factors of CoNS is their ability to form biofilms (Becker et al., 2014).

Biofilms are structured microbial communities embedded in a self-produced extracellular polymeric matrix (EPM) (Zhurina, 2014, Madigan, 2009), either attached to a surface or free floating as floccules. Many different bacterial species can form biofilms, and the composition of the matrix varies with strain and environmental conditions (Zhurina, 2014). A biofilm can consist of one or several species, and it has several protective advantages like resistance to physical force, toxins, antimicrobial agents and phagocytosis (Madigan, 2009). Since the bacterial cells in biofilms are so close, it also facilitates genetic exchange with a higher frequency than between planktonic bacterial cells (Madigan, 2009). Polysaccharide intercellular adhesin (PIA) is an important molecule in biofilm formation for many staphylococci, but for *S. haemolyticus* PIA only plays a minor role; proteins and extracellular DNA are more important (Fredheim et al., 2009). Fredheim and co-workers also showed high degrees of diversity in the biochemical profiles of *S. haemolyticus* biofilms, and that the genetic background for

biofilm formation is clearly different from what is commonly found in *S. epidermidis* (Fredheim et al., 2009).

Biofilm formation can schematically be described as a four-step process (Figure 1) (Otto, 2004, Becker et al., 2014, Heilmann, 2011). First the bacteria attach to a biotic or abiotic surface. The polymer surface of a medical device is abiotic. A biotic surface can be host tissue, or a medical device covered with host matrix proteins ("conditioning film"). Secondly the bacteria accumulate in multi-layered cell aggregates. In the third phase, the biofilm grows and matures, and the final step is detachment and dispersal of single cells or cell agglomerates. Several different factors can be involved in biofilm formation; both proteinaceous and nonproteinaceous surface- or surface associated molecules are described (Otto, 2004, Becker et al., 2014).

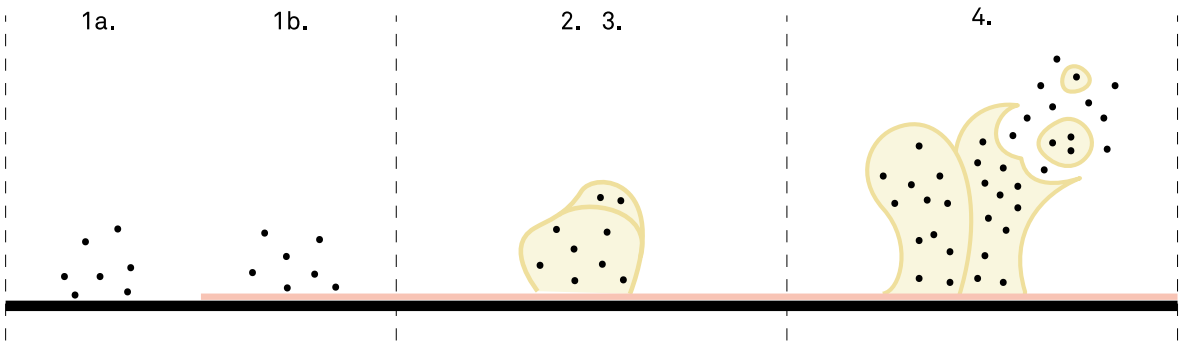


Figure 1 - Biofilm formation. Black dots are bacteria, beige is biofilm, black line is abiotic surface and red line is biotic surface. 1a: Adherence to abiotic surface, unspecific physiochemical interactions. 1b: Adherence to biotic surfaces (host factors / conditioning film), specific interactions. 2: Accumulation, intercellular adherence. 3: Maturation, production of extracellular biopolymers. 4: Detachment and dispersal. (Figure by Astrid Wolden)

2.4.6 Surface expressed proteins

Host tissue or host factors covering medical devices may serve as receptors for specific attachment of staphylococcal adhesins (Becker et al., 2014). Both non-covalently linked surface-associated proteins and covalently linked surface proteins can be important in binding the bacteria to biotic surfaces. The number of proteins varies among different staphylococcal species, and the expression can be altered by growth conditions (Foster et al., 2014). The number of open reading frames (ORFs) in *S. aureus* N315, *S. epidermidis* ATCC 12228 and *S. haemolyticus* JCSC 1435 are 2594, 2419 and 2678, respectively (Takeuchi et al., 2005).

CoNS can possess different surface-associated proteins, where one example is autolysin/adhesin Atl. Autolysin has an enzymatic function with hydrolysis of the cell wall peptidoglycan of the bacteria, leading to release of eDNA (Becker et al., 2014). Homologous proteins of autolysin with similar functions have different names in different species, like AtlE in *S. haemolyticus* (Takeuchi et al., 2005).

Cell wall anchored (CWA) proteins are covalently attached to the peptidoglycan layer of staphylococci. CWA proteins can bind to one or more human host factors, and one human factor can bind to several bacterial attachment factors. Not all ligands of CWA proteins are known (Becker et al., 2014, Foster et al., 2014). Foster and co-workers have suggested classifying CWA proteins into four groups based on their structure-function properties, where the most prevalent group is the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family (Foster et al., 2014). MSCRAMMs possess two adjacent IgG-like folds in their A domain that bind ligands by the "dock, lock and latch" (DLL) mechanism. In this mechanism the ligands dock to an open apo form, and when the structure changes into a closed form, the ligands are locked into place (Foster et al., 2014). A MSCRAMM subfamily in CoNS, called SesJ has been identified by Arora et al. (Arora et al., 2016). They found SesJ in *S. epidermidis*, and SesJ homologs in *S. haemolyticus* and other CoNS. They showed that the protein was expressed constitutively on the surface of a representative *S. epidermidis* strain, and thus SesJ is positioned to interact with protein targets in the environment, and can be important for CoNS virulence (Arora et al., 2016).

All CWA proteins contain a LPXTG motif (Leu-Pro-X-Thr-Gly; where X can be any amino acid) that anchor the protein to the cell wall (Foster et al., 2014). DLL was first described for SdrG/Fbe from *S. epidermidis* (Bowden et al., 2008, Ponnuraj et al., 2003). SdrG binds to fibrinogen, a protein found in blood (Herman et al., 2014). The Sdr protein subfamily of MSCRAMMs contains a serine-aspartate repeat region, and a region with IgG like folds, which bind ligands by the DLL mechanism (Foster et al., 2014, Becker et al., 2014). *Sdr* genes are also described in *S. haemolyticus* (Takeuchi et al., 2005).

Another family of CWA proteins is the SRRP family. Like The Sdr proteins, they have got a serine repeat region (SRR), but with alanine, valine or threonine instead of aspartate

(Arora et al., 2016). *sraP* from the SRRP family has been described in *S. haemolyticus* (Cavanagh et al., 2014, Takeuchi et al., 2005).

The biofilm-associated protein (Bap) is a surface protein of *S. aureus* that can mediate attachment and mediate accumulation of biofilm formation (Cucarella et al., 2001). *bap*-orthologous genes can also be found in CoNS, including *S. haemolyticus* (Potter et al., 2009, Tormo et al., 2005).

2.5 Fluorescence-activated cell sorting (FACS)

Fluorescence-activated cell sorters (FACS) are flow cytometers that can sort fluorescently labelled particles (e.g. eukaryotic or bacterial cells) by size and fluorescence from a mixed solution (Adan et al., 2017). When the laser beam hits the cells, the laser light is scattered in many directions. Forward scatter (FSC) is proportional to size of the particle and side scatter (SSC) indicates differences in the internal complexity of particles (BD-Biosciences, 2012, Adan et al., 2017). The cell sorting is based on electrostatic deflection of uniformly charged droplets, where uncharged droplets are collected in a waste container (Adan et al., 2017, BD-Biosciences, 2012).

When fluorochromes pass the laser beam they absorb photons, and the energy is released/emitted as light (fluorescence), which can be registered by a lens. The optimal excitation and emission wavelengths are within a specific range for each individual fluorochrome (Adan et al., 2017, BD-Biosciences, 2012).

2.6 Surface shaving of bacterial proteins

Surface proteins of pathogen bacteria might be important virulence factors, and knowing the proteins of the bacterial cell surface can be imperative combating infections. Functional genomics, such as proteomics, are important because the presence of genes in a genome does not say anything about when and to what extent the genes are expressed. Surface-attached proteins can be hard to work with because they have low abundance and many are quite insoluble compared to proteins within the cell. Surface protein shaving strategies are described below, and the technique used in this thesis is described in chapter 2.6.1 and 3.2.8.

The first-generation strategies for the identification of surface proteins combined two-dimensional gel electrophoresis followed by MALDI-TOF analysis. Membrane proteins are generally underrepresented in this technique compared to extracellular secreted proteins, due to their low solubility in most rehydration buffers (Olaya-Abril et al., 2014).

The second-generation proteomics is a gel-free approach, which combines digestion of live, intact cells with proteases in a liquid sample. The efficiency of the proteases can vary by accessibility of cleavage sites, abundance of substrate and the environment where the reaction takes place (Olaya-Abril et al., 2014). Surface-exposed moieties can be shaved and analysed by Liquid chromatography (LC) - Mass spectrometry (MS). This technique was originally described by Rodriguez-Ortega and co-workers for *Streptococcus pyogenes* to identify vaccine candidates (Rodriguez-Ortega et al., 2006).

Variations of the surface shaving approach have been described, and have been performed in a range of species (Olaya-Abril et al., 2014). The digestion time of the protease can be optimized for each experiment. The original approach had a short digestion with the protease (trypsin) (Rodriguez-Ortega et al., 2006). It is also possible to introduce several digestion steps. Tjalsma and co-workers did a modification of the original protocol with a redigestion step of the already generated surfome of the *Bacillus subtilis* model organism (Tjalsma et al., 2008).

Trypsin is a widely used protease in surface shaving approaches because it works well in a solution, cleaves at specific sites, and its specificity generates peptide fragments where the length is ideal for MS analysis (Olaya-Abril et al., 2014). However, using trypsin means that loops without a free end need two cleavage sites to generate a peptide, and both sites must be accessible to the protease. Therefore, an unspecific protease, proteinase K, can be included in the original protocol. However, the reproducibility will be low due to non-specific cleavage, and the quality of the MS/MS spectra is generally lower than for tryptic peptides (Olaya-Abril et al., 2014). Nevertheless, some surface proteins are only found after proteinase K treatment and not with trypsin only (Hilleringmann et al., 2009).

Tjalsma and co-workers have described a “shedding and shaving” technique (Tjalsma et al., 2008). Proteinases are able to penetrate the cell wall by passive diffusion, possibly

causing destabilisation of cells and release of cytoplasmic proteins. Immobilized proteinases can be used to avoid this. Tjalsma et al. used immobilized trypsin to shave *B. subtilis* and compared the results with the digestion by free trypsin ("shedding and shaving") (Tjalsma et al., 2008). In general, fewer proteins were identified from the immobilized trypsin treatment and predicted cytoplasmic proteins were identified in both treatments. This indicates that the trypsin protease is not the cause of released cytoplasmic proteins due to possible destabilisation of cells (Tjalsma et al., 2008, Olaya-Abril et al., 2014).

There are at least three hypotheses that might explain the presence of predicted cytoplasmic proteins when performing bacterial surface shaving; i) cellular lysis; ii) cytoplasmic proteins has reached the surface by the secretory pathways (moonlighting proteins); iii) cytoplasmic proteins are released by shedding membrane-vesicle (MV) structures (Olaya-Abril et al., 2014, Solis and Cordwell, 2016). If cells are in exponential phase of growth and undergo active division, the rate of cell death is low. A significant reduction of cell viability after protease treatment has never been found (Olaya-Abril et al., 2014). Several species produce membrane vesicles, and they have also been described in staphylococci. Release of MVs in incubation buffer after culturing and washing cells might explain the identification of predicted cytoplasmic proteins (Olaya-Abril et al., 2014). *S. aureus* produces MVs associated with the bacterial surface or released into the surrounding environment depending on bacterial growth conditions (Askarian et al., 2018). The rate of predicted cytoplasmic proteins in bacteria when performing surface shaving is extremely variable (e.g. 0%-70%), also when performing surface shaving of the same specie (Olaya-Abril et al., 2014).

A false-positive control strategy for Gram-positive cell surfaceomics has been suggested by Solis and Cordwell to better control for cell lysis and the release of intracellular proteins (Solis and Cordwell, 2016, Solis et al., 2010). Whole cell fractions have a short incubation in isotonic buffer with a protease (e.g. trypsin) before released peptides are collected and analysed by LC-MS/MS. The false positive control is incubated as the sample, but with no protease included (Solis and Cordwell, 2016, Solis et al., 2010). The false-positive control strategy improved enrichment of surface-exposed peptides for *S. aureus* in the trypsin data set to approximately 80% (Solis et al., 2010).

A relative quantitative cell surface proteome profiling of *S. aureus* has been done by using a combination of $^{14}N^{15}N$ metabolic labelling, biotinylation and LC-MS/MS approaches (Hempel et al., 2011, Hempel et al., 2010). Biotinylation can be used as a selective labelling method for surface exposed proteins, and it is possible to specifically biotinylate one single protein (Elia, 2008).

A variation of the surface shaving technology has been described where host proteins interact with bacterial surface proteins. The binding of human host serum proteins to *S. aureus* cells has been profiled by surface shaving with trypsin and MS analysis of liberated peptides (Dreisbach et al., 2011). Host proteins interacting with surface proteins of *Streptococcus gallolyticus* has been described; a preincubation step where bacterial cells were allowed to capture human proteins from epithelial cell lysates were applied (Boleij et al., 2011). To our knowledge, an analysis of surfaced proteins of bacteria expressed during direct colonisation of human cells has never been described.

2.6.1 The Lipid-based Protein Immobilization (LPI™) technology

Lipid-based Protein Immobilization (LPI™) is a recently developed technology, and the method used for surface shaving in this thesis. The technology is based on immobilization of biological material (plasma membrane, mitochondria, bacteria etc.) within a flow cell, followed by digestion of exposed proteins by an enzyme, such as trypsin (Figure 2) (Karlsson et al., 2009, Karlsson et al., 2012, Karlsson et al., 2014, Karlsson et al., 2016, Jansson et al., 2012, Gonzales-Siles et al., 2017). By immobilizing intact bacteria in the flow cell and performing limited digestion, surface shaving of the exposed proteins can be achieved.

The LPI approach differs from traditional methods by retaining surface proteins in their native compartment, not trying to solubilize the proteins (Sui et al., 2011). No detergents are required and no sample clean up is needed prior to downstream analysis. The environment around the proteins can be changed without loss or dilution of the sample, and multiple digestion steps can be applied to increase sequence coverage by changing the protease solution in the flow cell (Sui et al., 2011).

As reported in a study aimed at performing proteomic typing of *Helicobacter pylori* strains using the LPI™-technology, 60% of the strain-specific peptides that were found

to be unique biomarkers of the *H. pylori* J99 strain belonged to membrane associated proteins (Karlsson et al., 2012).

To enable comparison between samples, different protein labelling methods can be utilised for quantitative analysis, for example labelling of peptides with stable isotopes, either during culturing (Stable isotope labelling with amino acids in cell culture, SILAC), prior to digesting samples (Isotope-coded affinity tags, ICAT) or post-digestion (Isobaric tags for relative and absolute quantitation, iTRAQ, and tandem mass tags, TMT) (Collier et al., 2010). The TMT-labelling enables relative quantification with good sensitivity between peptide samples that are uniquely labelled and thereafter pooled for Mass Spectrometry (MS) analysis (Thompson et al., 2003). MS/MS-based tag detection such as Liquid chromatography (LC)-MS is often used to perform comparative quantitative proteomic profiling between two cell lines (Paulo et al., 2013).

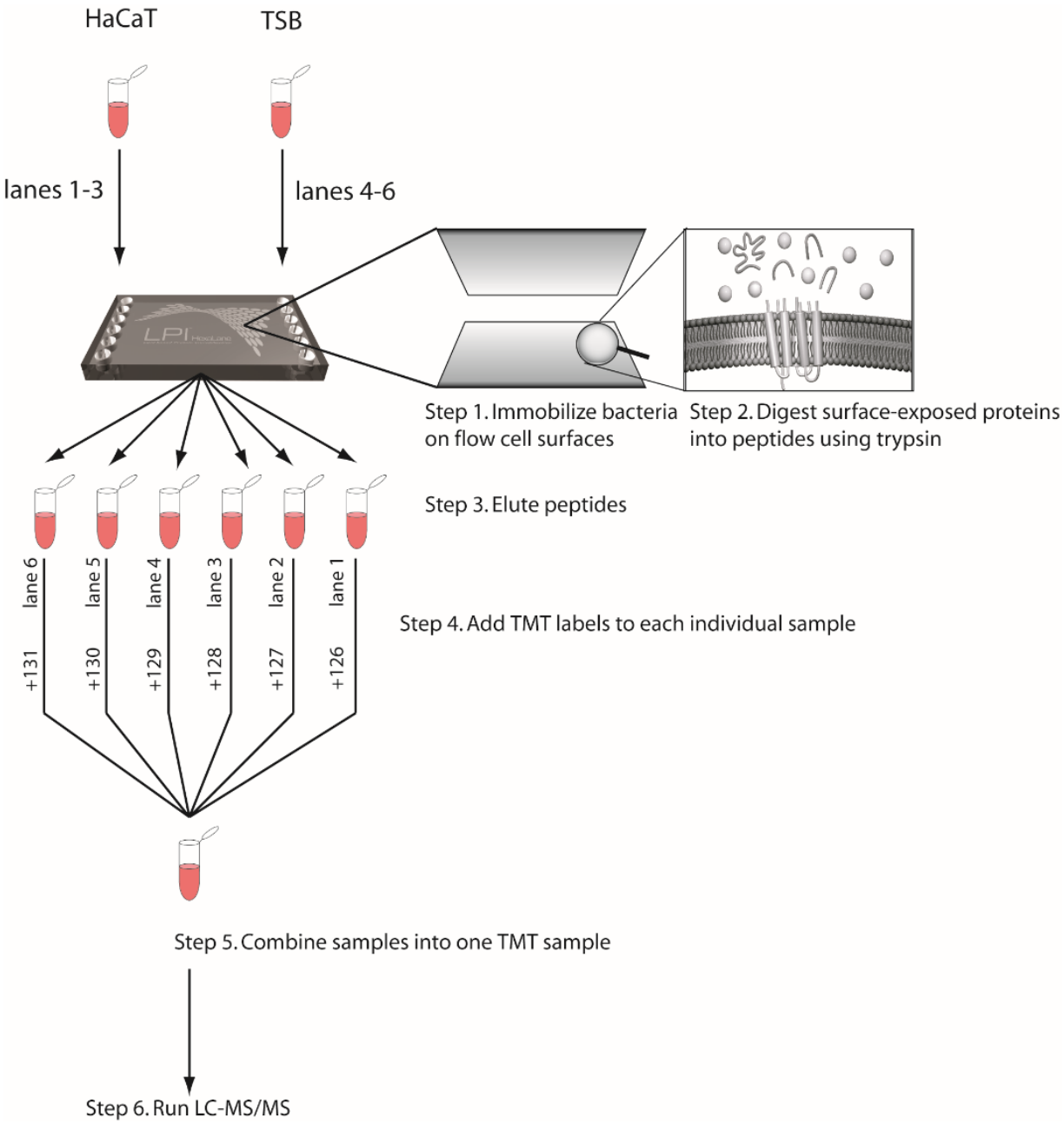


Figure 2 –Overview of the workflow for sample processing and peptide release by LPI™ HexaLane (Nanoxis Consulting AB). Prepared bacterial suspension is loaded into the LPI™ HexaLane Flow Cell, with three biological replicates for each condition (step 1), enzymatic digestion of bacterial proteins is performed by using trypsin (step 2) and peptides released in the digestion step are eluted (step 3). Each individual digested sample in a set is labelled with TMT tag (step 4) before they are pooled (step 5) for LC-MS/MS analysis (step 6) (Figure by Nanoxis).

3 Material and method

3.1 Introduction

The material and method section describes the development of a surface shaving method for determining which surface proteins are expressed by *S. haemolyticus* during colonisation of human keratinocytes (HaCaT cells).

The section is divided into five parts. The first part describes standard lab techniques used repeatedly during method development (3.2), followed by preliminary experiments in preparation of the major surface shaving experiments (3.3) before running the initial surface protein shaving experiment (3.4), optimizations of the methods used in the initial experiment (3.5) and the optimized surface protein shaving experiment (3.6). Bacterial and eukaryotic sample preparation and cell sorting on FACS were performed at UiT The Arctic University of Norway. Proteomic analysis was performed by Nanoxis Consulting AB, Sweden.

3.2 Repeatedly used techniques

3.2.1 *Staphylococcus haemolyticus* strains

In the first experiments, a community strain of *S. haemolyticus* with identification number 57-26 was used to test the methods (Cavanagh et al., 2016).

Before the initial surface shaving experiment was performed, the experimental strain was changed to *S. haemolyticus* with identification number 53-38 (ERS066380). This is a clinical wound sample (Cavanagh et al., 2014) with known high level of adhesion to human cells (unpublished results). The genome of the strain possesses 2537 Coding DNA Sequences (CDS) for proteins.

3.2.2 Cultivation of *S. haemolyticus*

When growing *S. haemolyticus*, both primary and secondary cultures were made (procedure below). Secondary cultures (subcultures) were grown to specific OD_{600} values.

Tryptic Soy Broth (TSB) is a general-purpose broth medium for the cultivation of fastidious microorganisms. TSB was used when growing primary cultures of *S. haemolyticus*.

Dulbecco's Modified Eagle's Medium (DMEM) with 10% Heat Inactivated Fetal Bovine Serum (FBS) is normally used to cultivate mammalian cells. The media was tested and used as secondary bacterial culture medium for *S. haemolyticus* in most of the experiments to avoid changing the media for the bacteria as they were added to the mammalian cells (HaCaT). TSB or DMEM with 10% FBS and various concentrations of TSB were also used as secondary culture medium, depending on the experiment. Readouts were OD at 600nm and/or determination of CFU by plating.

Table 2 –Reagents and instruments for preparing primary and secondary bacterial cultures.

Reagents and instruments	**Firm**	**Catalogue no.**
Dulbecco's Modified Eagle's Medium (DMEM) – high glucose	Sigma-Aldrich/Merck	D5796-500ml
Heat Inactivated FBS Fetal Bovine Serum	ThermoFisher Scientific	10500-064, 500ml
Tryptic Soy Broth (TSB)	Media production, UNN (BD, Merck)	• 211825 (TSB, BD) • 101614 (Agar agar, Merck)
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich/Merck	• D8537-500ml
Blood agar plates	Media production, UNN (Oxoid)	• CM0271 (Blood Agar Base No. 2) • SR0051E (Defibrinated sheep blood)
Shake Incubator, Incu-Shaker 10LR	Benchmark	NA
Ultrospec 2000 spectrophotometer 80-2106-00	Pharmacia Biotech	NA

3.2.2.1 Primary cultures

Strains stored at -80 °C were transferred to a blood agar plate and incubated at 37 °C overnight. One colony of *S. haemolyticus* from a blood agar plate was used to inoculate 5 ml TSB, and shaken vigorously (220-250 rpm) at 37 °C for 10 hours to overnight (stationary phase).

3.2.2.2 Secondary cultures (subculturing)

- The TSB culture was diluted 1:100 or 1:200 depending on the experiment.

- TSB, DMEM with 10% FBS or DMEM with 10% FBS and various concentrations of TSB were used when diluting primary cultures to secondary cultures.
- Sample was shaken vigorously (220-250 rpm) at 37 °C, with incubation times depending on the experiment and media used (details given under each experiment below). Bacteria have different growth curves depending on the growth medium.
- OD_{600} measurements and/or determination of CFU/ml were performed at different time points.
- The expression of CWA proteins can be altered by growth conditions (Foster et al., 2014). Based on previous experiments with surface expressed proteins (personal communication), bacterial cultures were grown to mid/late exponential phase (Askarian et al., 2016).
- Overnight cultures were washed twice with PBS at 4500xG, and resuspended to various concentrations in DMEM with 10% FBS, TSB or PBS, depending on the experiment.
- DMEM with 10% FBS was used for samples that were going to be incubated with HaCaT cells, and were diluted to a concentration of $4x10^7$ bacteria/ml before the solution was added to the HaCaT cells, multiplicity of infection (MOI) 1:100.
- Samples not incubated with HaCaT cells, but sorted on FACS were diluted 1:3 before running on FACS (sample dependent).

3.2.3 Eukaryotic human keratinocyte HaCaT cells

HaCaT cells from a human keratinocyte cell line (Boukamp et al., 1988) (Cell Lines Service, Germany, no. 300493) were used in the experiments.

Handling of HaCaT cells, e.g. thawing of frozen cells and subculturing, were done according to the recommendations of CLS Cell Lines Service.

Table 3 - Reagents and equipment for subculturing of HaCaT cells.

Reagents/ equipment	**Firm**	**Catalogue no.**
Dulbecco's Modified Eagle's Medium (DMEM) – high glucose	Sigma-Aldrich/Merck	D5796-500ml
Heat Inactivated FBS - Fetal Bovine Serume	ThermoFisher Scientific	10500-064, 500ml
Penicillin-Streptomycin	Sigma-Aldrich/Merck	P0781-100ml
Trypsin-EDTA Solution	Sigma-Aldrich/Merck	T4049-100ml
PBS with 0.25 mM EDTA	NA	NA
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich/Merck	D8537-500ml
Nunc™ EasYFlask™ Cell Culture Flasks, T75, filter	ThermoFisher Scientific	156499
Cell counter - Scepter Handheld Automated Cell Counter	Millipore Corporation	PHCC00000
Scepter Sensors – 60 µm	Millipore Corporation	PHCC60050
12 Well tissue culture plates, Falcon™, Polystyrene, Flat Bottom	ThermoFisher Scientific	353043
6 Well tissue culture plates, Falcon™, Polystyrene, Flat Bottom	ThermoFisher Scientific	353046

3.2.3.1 Subculturing of HaCaT cells

- HaCaT cells were picked from the nitrogen tank, thawed and grown to 80-90% confluence in DMEM with 10% FBS and Penicillin-Streptomycin in T75 cell culture flasks at 37 °C and 5% CO_2.
- Cells were washed with 10 ml PBS, treated with 2.5 ml PBS with 0.25 mM EDTA for 10 minutes and 2.5 ml Trypsin-EDTA solution for 1-3 minutes at 37 °C and 5% CO_2.
- Cells were resuspended in DMEM with 10% FBS and Penicillin-Streptomycin to a total volume of 10 ml. Penicillin-Streptomycin was not used if *S. haemolyticus* was intended to grow in the cell culture medium.
- An appropriate volume of cell suspension was added to DMEM with 10% FBS (and Penicillin-Streptomycin) in a T75 cell culture flask.
- Cells were incubated at 37 °C and 5% CO_2 to 80-90% confluence.

3.2.3.2 Transferring HaCaT cells to tissue culture plates

HaCaT cells were transferred to tissue culture plates before adding bacteria. 12 well plates were used in the initial surface shaving experiment, and 6 well plates in the optimized experiment.

- HaCaT cells were grown to 80-90% confluence and loosened from the cell culture flask, as described above.
- Cells were resuspended in DMEM with 10% FBS to a total volume of 10 ml.

- A cell counter was used to calculate the cell concentration.
    - 10 µl of the cell suspension was transferred to a tube with 90 µl PBS.
    - 3 tubes/measurements were made for each sample.
- The suspension was diluted to a concentration of $2x10^5$ cells/ml in order to obtain a MOI of 1:100 when adding $4x10^7$ bacteria. An example of calculating the concentration of the suspension is given in the appendix.
- To a 12 well tissue culture plate, 2 ml of the $2x10^5$/ml cell suspension was added to each well, and to a 6 well plate, 4 ml was added to each well.
- Incubation of plates were done at 37 °C and 5% CO_2 overnight.

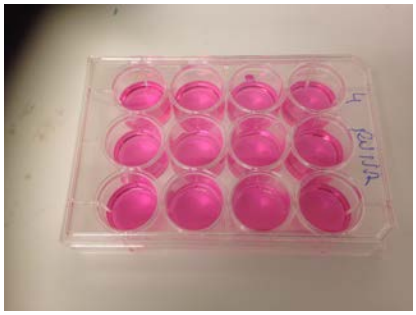


Figure 3 - 12 well tissue culture plate and HaCaT cells grown in DMEM with 10% FBS

3.2.4 Incubating *S. haemolyticus* with HaCaT cells

- *S. haemolyticus* were added to HaCaT cells grown to confluency.
- Tissue culture plates were microscoped to check if the growth was confluent, and the cell culture medium was removed from the HaCaT cells.
- Washed and resuspended *S. haemolyticus* culture (3.2.2.2) in DMEM with 10% FBS was added to the tissue culture plate. 1 ml of culture was added to each well in 12 well plates, and 2 ml of culture was added to 6 well plates.
- Plates were centrifuged at 900xG for 10 minutes at 37 °C, and then incubated at 37 °C and 5% CO_2 for 50 minutes.
- The centrifugation step of the tissue culture plates was implemented in the optimized surface shaving experiment. Before that, the plates were incubated at 37 °C and 5% CO_2 for 1 hour.

3.2.5 Removing eukaryotic and bacterial cells from tissue culture plates

Mechanical detachment of eukaryotic and bacterial cells from the tissue culture plates with a cell scraper followed by pipetting was done before separation on FACS (Figure 4).

Table 4 - Reagents and equipment for removing cells from wells from tissue culture plates.

Reagent / equipment	**Firm**	**Catalogue no.**
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich/Merck	D8537-500ml
Cell scraper	VWR	734-2602
Corning™ Falcon™ Test Tube with Cell Strainer Snap Cap, 5 ml Polystyrene Round-Bottom Tube	ThermoFisher Scientific	352235

- After incubation of *S. haemolyticus* with HaCaT cells (3.2.4), excess bacterial suspension was aspirated from the tissue culture plates and washed 4 times with PBS to remove free floating bacterial cells.
- 12 well plates: 200 µl of PBS was added to each well and a cell scraper was used to loosen cells. A balloon was used to press the liquid through the filter when transferring the sample to 5 ml polystyrene tubes with cell strainer cap.
- 6 well plates: 400 µl of PBS was added to each well and a cell scraper was used to loosen cells. A balloon was used to press the liquid through the filter when transferring the sample to 5 ml polystyrene tubes with cell strainer cap. 100 µl of PBS was used to flush the wells after scraping and added to the same tube.

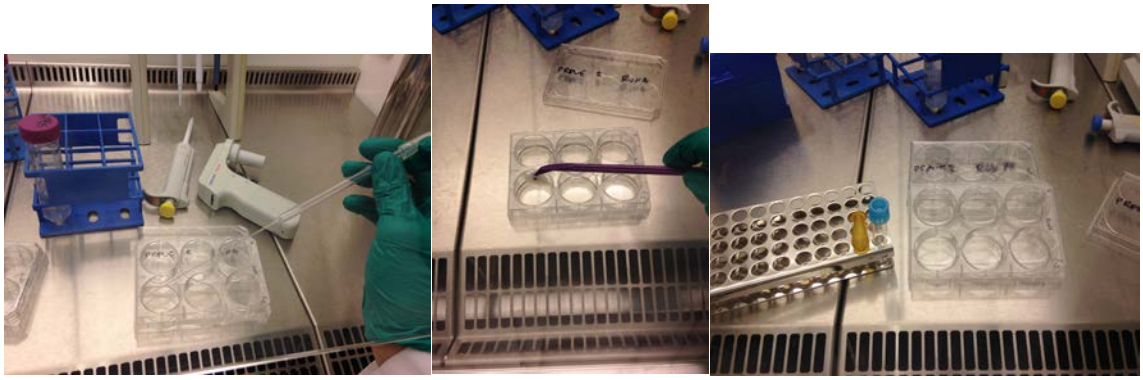


Figure 4 – Removal of cells from tissue culture plates. Aspiration of PBS from a 6 well tissue culture plate (LEFT) before using a cell scraper to remove HaCaT and S. haemolyticus cells (MIDDLE) and transferring to a 5 ml polystyrene tube with cell strainer cap using a balloon (RIGHT).

3.2.6 Fluorescence-activated cell sorting system (FACS)

S. haemolyticus was sorted from HaCaT cells using the Fluorescence-activated cell sorting system, FACS Aria III, at the Bio-imaging Platform at IMB, UiT. Settings for

sorting bacteria were determined by test runs before the initial surface protein shaving experiment.

Table 5 - Instruments and equipment when running FACS.

Instrument / equipment	**Firm**	**Catalogue no.**
FACSAria III Cell Sorter	BD	NA
Polystyrene Particle Size standard	Spherotech	PPS-6K
Nano Fluorescent Size Standard Kit	Spherotech	NFPPS-52-4K
Vancomycin BODIPY™	ThermoFisher Scientific	V34850

- Fluorescent beads of known sizes were used to calibrate and adjust the instrument in order to sort particles with the size of staphylococci (Figure 5 and 6).
- The Vancomycin BODIPY™ FL conjugate (excitation/emission maxima ~503/512 nm) is a fluorescent dye targeting the Gram-positive bacterial cell wall. The dye was added to the *S. haemolyticus* suspension before running FACS to be able to sort the bacteria from other non-fluorescent particles of the same size. 30 µl of 100µg/ml to each 5 ml sample was used in the optimized surface shaving experiment.
- Vancomycin BODIPY™ was excited with a 488nm blue laser. A FITC-detector was used to read the emitted, green, fluorescent light.
- Normal density filter 1.0 was used in front of the FSC detector.

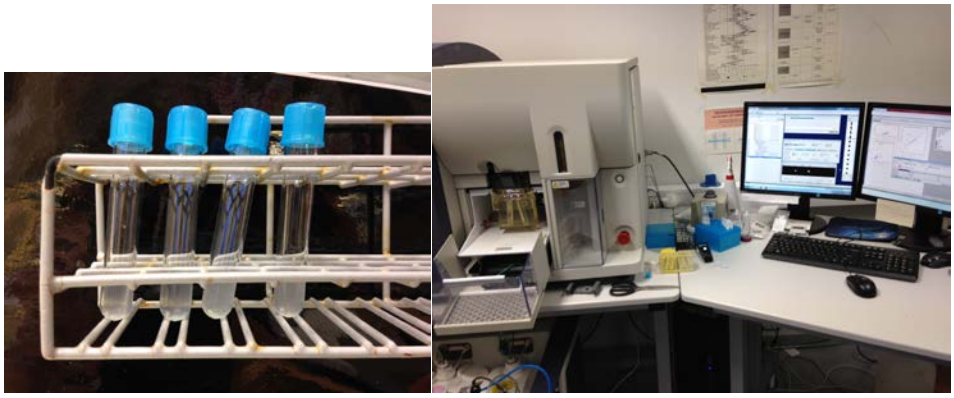
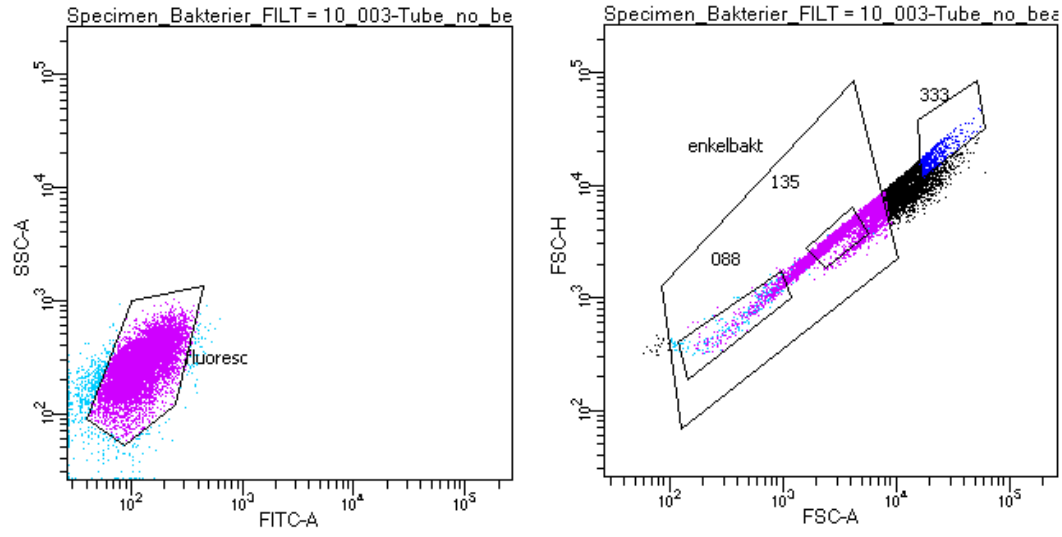


Figure 5 – Setup for FACS. Samples in 5 ml polystyrene round-bottom tubes with cell-strainer cap (LEFT). The FACS Aria III Cell Sorter and computer screen (RIGHT).



Tube: Tube_no_beads

Population	#Events	%Parent	%Total
All Events	14,130	####	100.0
135	4,558	32.3	32.3
333	556	3.9	3.9
088	564	4.0	4.0
enkelbakt	10,000	70.8	70.8
fluoresc	9,324	93.2	66.0

Figure 6 – Example of scatter plot from FACS Aria III software BD FACSDiva 8.0.1 in the optimized surface protein shaving experiment to show how settings for sorting particles were determined based on size and fluorescence. RIGHT window: Size of particles sorted on FACS. The three smallest gates represents the area where standard fluorescent beads of 0.88, 1.35 or 3.33 µm could be found. To be able to find single/doublettes/quadruplets of staphylococci, the area for sorting partidcles of interest was set as shown with the largest gate. The area to the right of the gate was believed to be cell debris from HaCaT cells or possibly larger clumps of bacteria. LEFT window: Particles were sorted by fluorescent signal strength. A FITC-detector was used to read the green emitted BODIPY™ fluorescent light.

3.2.7 Determination of CFU: Serial dilutions and plating

Colony forming units (CFU)/ml was determined by making serial dilutions and plating on blood agar plates.

Table 6 - Reagents used for serial dilutions and plating on blood agar plates.

Reagent	**Firm**	**Catalogue no.**
Blood agar plates	Media production, UNN (Oxoid)	• CM0271 (Blood Agar Base No. 2) • SR0051E (Defibrinated sheep blood)
0.85% NaCl	Media production, UNN (Merck)	• 106404 (NaCl)

- Samples were diluted to 10^{-6} in 0.85% NaCl (20 µl to 180 µl) in 96 well plates (Figure 7).

- Parallel dilutions were made and plated where adequate sample material was available.
- 25 µl of all the dilutions and the stock solution were transferred to two blood agar plates with a multi channel pipette. Plates were tilted so that droplets were allowed to run down the blood agar plate making a line of bacterial inoculum (Figure 7).
- Plates were incubated at 37 °C overnight.
- CFU was counted and CFU/ml was calculated: $\frac{CFU}{ml} = \frac{CFU\ x\ dilution\ factor}{volume\ on\ agar\ plate}$

  Example: 145 colonies counted on 10^{-4} dilution: $\frac{145\ x\ 10\text{^}4}{0.025\ ml} = 5.8\ x\ 10^7 CFU/ml$
- The standard deviation (SD) (σ) was calculated where parallel dilutions were made (square root of the variance).

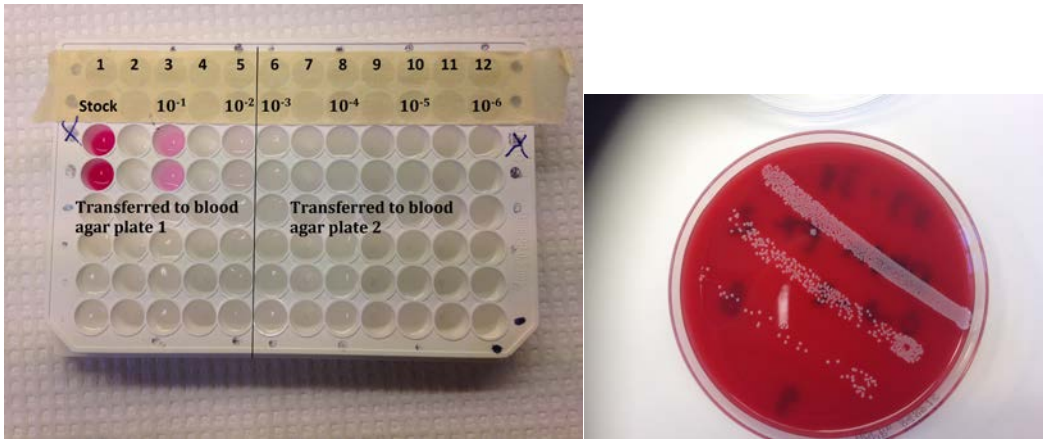


Figure 7 – Serial dilution and plating. LEFT: 96-well bacterial dilution plate, column 1-12 and row 1-6. One row with dilutions for one sample. Stock, 10^{-1} and 10^{-2} dilutions were transferred to blood agar plate 1, and 10^{-3}-10^{-6} dilutions to blood agar plate 2. RIGHT: Example of a plated serial dilution after incubation of the blood agar plate (stock, 10^{-1} and 10^{-2} dilution). The densest growth is the stock solution.

3.2.8 LPI™ Flow Cell and proteomics

Resuspended pellets from centrifugation after FACS were delivered to Nanoxis for surface shaving and proteomics (method below) (Figure 8). The difference of the proteomics done in a general protein analysis and a relative quantification study is the use of TMT tags, otherwise the methods are similar.

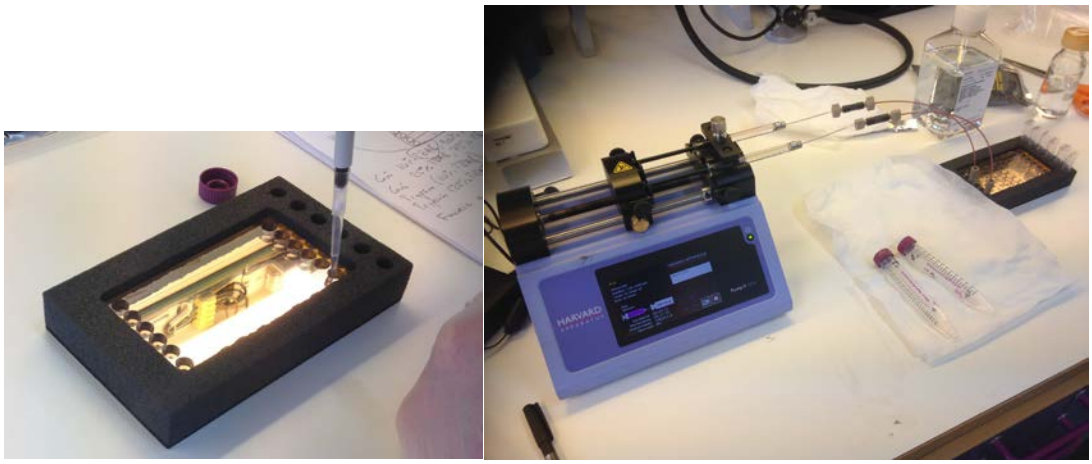


Figure 8 - The LPI™ HexaLane Flow Cell (golden colour) with syringe pump.

3.2.8.1 Sample processing and generation of peptides by LPI™ HexaLane

After FACS and concentration in a centrifuge, the samples were immediately loaded into the LPI™ HexaLane Flow Cell (Nanoxis Consulting AB), as seen in Figure 2, step 1. An excess of bacteria (45 µl) was applied to the flow cell to fill the LPI™ Flow Cell channel, which has a volume of approx. 35 µl. The immobilized bacteria were incubated for 35 minutes at room temperature, to allow bacterial attachment, and the channels were subsequently washed with 200 µL of PBS buffer using a syringe pump (Harvard Apparatus) to remove excess, unbound bacteria. The syringe pump used was operating at a flow rate of 50 µL/min. Enzymatic digestion of bacterial proteins was performed by injecting 100 µL of trypsin (Initial surface shaving experiment: 20 µg/ml trypsin in PBS with 10% TEAB; Optimized surface shaving experiment: 40 µg/mL trypsin in PBS) into the LPI HexaLane FlowCell channels, using the same syringe pump setup. Excess trypsin was removed from the ports and the flow cell was left for incubation for 20 min. at room temperature. After digestion, the peptides created during the digestion step were eluted with 200 µL PBS using the syringe pump and subsequently acidified using 4 µL formic acid (neat) to stop the digestion. The peptide samples were centrifuged for 10 minutes at 10000xg, and transferred to a new sample tube. Samples were dried using a SpeedVac (Eppendorf) and then stored at -20 degrees Celsius until preparation for Mass Spectrometry analysis. The digested samples were resuspended in 0.5 M TEAB (Triethylammonium Bicarbonate) prior to labelling with the TMT® according to the manufacturer's instructions (ThermoFisher Scientific). In a set, each sample was labelled with a unique tag from a TMT 6plex isobaric mass tag labelling kit. After TMT labelling, the samples in a set were pooled.

3.2.8.2 Mass Spectrometry (MS)

Samples were reconstituted with 15 µL of 0.1% formic acid (Sigma-Aldrich/Merck) in 3% acetonitrile (Sigma-Aldrich/Merck) and analysed on a QExactive (ThermoFisher Scientific) mass spectrometer interfaced to an Easy-nLC II (ThermoFisher Scientific). Peptides (2 µL injection volume) were separated using an in-house constructed analytical column (200 × 0.075 mm I.D.) packed with 3 µM Reprosil-Pur C18-AQ particles (Dr. Maisch, Germany). Solvent A was 0.2% formic acid in water and solvent B was 0.2% formic acid in acetonitrile. The following gradient was run at 200 nL/min; 5–30% B over 75 min, 30–80% B over 5 min, with a final hold at 80% B for 10 min. Ions were injected into the mass spectrometer under a spray voltage of 1.6 kV in positive ion mode. The MS scans was performed at 70 000 resolution (at m/z 200) with a mass range of m/z 400–1800 for the QExactive, respectively. MS/MS analysis was performed in a data-dependent mode, with the top ten most abundant doubly or multiply charged precursor ions in each MS scan selected for fragmentation (MS/MS) by stepped high energy collision dissociation (stepped HCD) of NCE-value of 25, 35 and 45. For MS/MS scans the resolution was 35,000 (at m/z 200) for the QExactive with a mass range of m/z 100–2000. The isolation window was set to 1.2 Da, intensity threshold of 1.1e4 and a dynamic exclusion of 30 s, enabling most of the co-eluting precursors to be selected for MS/MS.

3.2.8.3 Database search for protein TMT quantification

For relative quantification and identification the MS raw data files for each TMT set were merged in the search using Proteome Discoverer version 1.4 (ThermoFisher Scientific). A database search for each set was performed with the Mascot search engine (Matrix Science LTD) using species-specific databases downloaded from Uniprot. The data was searched with MS peptide tolerance of 5 ppm for Q-Exactive runs and MS/MS tolerance for identification of 100 millimass units (mmu). Tryptic peptides were accepted with 1 missed cleavage and variable modifications of methionine oxidation, cysteine methylthiolation and fixed modifications of N-terminal TMT6plex and lysine TMT6plex were selected. The detected peptide threshold in the software was set to 1% FDR (false discovery rate) for the experiments performed on the QExactive, by searching against a reversed database.

Identified proteins were grouped by sharing the same sequences to minimize redundancy. For TMT quantification, the ratios of the TMT reporter ion intensities in MS/MS spectra (m/z 126–131) from raw data sets were used to calculate fold changes between samples. Ratios were derived by Proteome Discoverer using the following criteria: fragment ion tolerance as 80 ppm for the most confident centroid peak and missing values are replaced with minimum intensity. TMT reagent purity corrections factors are used and missing values are replaced with minimum intensity. Only peptides unique for a given protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family. The quantification was normalized using the protein median. The results were then exported into MS Excel for manual data interpretation and statistical analysis.

For the statistical analysis, first, proteins displaying more than 20% variation between the individual LPI channels for the three study group and the three control channels respectively were removed. This was done by calculating the ratio of the separate TMT-labels in a group, and the average of the combined channels e.g. 126/(average 126 + 127 + 128). Proteins with ratios between 0.8 and 1.2 were included in the protein list. Second, a Welch's t-test was performed (3 technical replicates vs. 3 technical replicates) and only proteins passing filter $p < 0,05$ was accepted. Third, a fold change of at least 1.5 was set as a threshold to list proteins that had a relevant up or down regulation.

3.3 Preliminary work

3.3.1 Optimization of growth media for *S. haemolyticus*

Growth of *S. haemolyticus* in cell culture medium, DMEM with 10% FBS, instead of conventional TSB was investigated to avoid changes in expression of bacterial proteins related to the culture media.

S. haemolyticus was grown in TSB or DMEM with 10% FBS and 5 or 10% TSB added to the medium in a 96-well microplate, OD_{600} was measured every 15 minutes for 24 hours by a Synergy H1 Hybrid reader (BIOTEK, Software Gen5). After the microplate experiment, the growth in DMEM with 10% FBS and various concentrations of TSB was tested to see if TSB had to be added to the cell culture medium to make *S. haemolyticus* grow. A primary TSB culture (3.2.2.1) of *S. haemolyticus* was diluted 1:100 in DMEM

with 10% FBS and various concentrations of TSB added (0%, 5% or 10%). Samples were shaken vigorously (250 rpm) at 37 °C and OD_{600} was measured at different time points. CFU/ml was determined (3.2.7) for the growth of *S. haemolyticus* 53-38 in DMEM with 10% FBS.

3.4 Surface shaving of expressed bacterial proteins, initial experiment

Surface shaving of expressed *S. haemolyticus* cell surface proteins with the LPI™ Flow cell was done twice; once in the initial experiment (this chapter) and once in the optimized experiment (3.6).

Three different sets of samples were set up in three parallels and run on the LPI™ Flow Cell by Nanoxis. Sample description in Table 7 below.

Table 7 - Overview of samples for S. haemolyticus surface shaving of proteins in initial experiment.

Type of sample	**Number**
• Secondary culture of *S. haemolyticus* 53-38 in cell culture medium DMEM with 10% FBS • **Incubated with** HaCaT cells • Separated on FACS	**Sample 1-3**
• Secondary culture of *S. haemolyticus* 53-38 in TSB • **Not incubated with** HaCaT cells • Separated on FACS	**Sample 4-6**
• Primary culture of *S. haemolyticus* 53-38 in TSB • Not incubated with HaCaT cells • Not separated on FACS	**Sample 7-9**

Sample 1-3: Secondary culture (1:200 dilution from primary culture) DMEM with 10% FBS (3.2.2), was incubated for 6 hours and 35 minutes to OD_{600} 0.6.

Sample 4-6: Secondary culture (1:100 dilution of primary culture) in TSB (3.2.2) was incubated for 2 hours and 30 minutes to OD_{600} 0.9.

Sample 7-9: Primary TSB cultures (3.2.2.1) were analysed directly on the LPI™ Flow Cell after incubation. The bacterial concentration (CFU/ml) of samples 7-9 was higher than for samples 1-6 when transferring them to the LPI™ Flow Cell.

Secondary cultures (sample 1-6) were washed in a fixed angle centrifuge (Eppendorf 5430 R) (3.2.2.2). Samples 1-3 were resuspended in DMEM with 10% FBS (ready for HaCaT), and sample 4-6 in PBS (ready for FACS).

One 12 well tissue culture plate with HaCaT cells was used for each sample (sample 1-3) (3.2.3.2). Tissue culture plates with eukaryotic cells were incubated for 16 hours before adding bacteria (3.2.4) and preparing samples (3.2.5) prior to cell sorting on FACS (3.2.6).

3.4.1 FACS and centrifugation (samples 1-6)

After sorting on FACS (3.2.6), samples were centrifuged at 6000xG in a fixed angle benchtop centrifuge (Eppendorf 5430 R). Supernatant was removed and the pellet was resuspended in the remaining liquid.

3.4.2 LPI™ Flow cell and proteomics (sample 1-9)

A TMT tag was used for *S. haemolyticus* samples 1-6 to do a relative quantification study and to evaluate up- and down-regulation of proteins, and a general protein analysis was done for samples 7-9 (3.2.8).

3.5 Optimization of the initial bacterial protein surface shaving experiment

In the initial surface shaving experiment, the concentration of bacteria added to the LPI™ Flow Cell was too low, and no results with the TMT tag protein analysis were obtained. Optimizations were made before repeating the surface protein shaving experiment, and the goal was to increase the bacterial concentration after FACS before inoculating the LPI™ Flow Cell.

3.5.1 Conditions of the LPI™ Flow Cell

Nanoxis ran a protein analysis with different trypsin standards (PBS 20 μg/ml trypsin, PBS + 10% TEAB, PBS 40 μg/ml trypsin), to see what kind that gave least bacterial lysis.

3.5.2 Survival in PBS after centrifugation

The survival of *S. haemolyticus* in PBS was tested for up to 70 minutes after FACS and centrifugation by determining CFU/ml (3.2.7).

3.5.3 Optimization of centrifugation

Centrifugation of **secondary cultures** of *S. haemolyticus* (3.2.2.2) was changed from a fixed angle (Eppendorf 5430 R) to a swing bucket benchtop centrifuge (Beckman Coulter Allegra X-15R) to achieve higher concentration of bacterial cells (Figure 9).

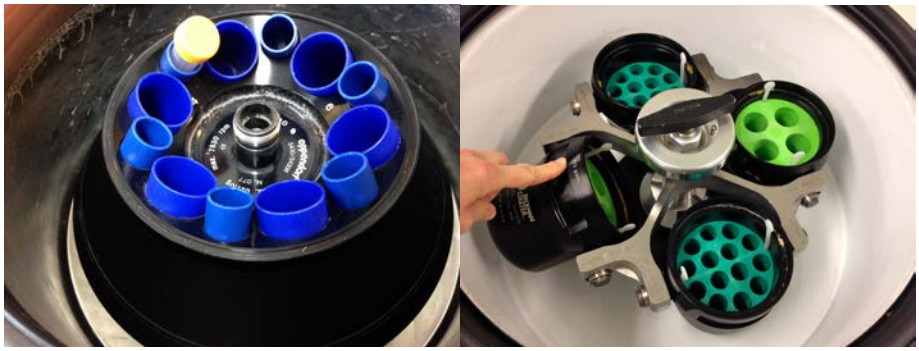


Figure 9 - Fixed angle rotor in Eppendorf 5430 R benchtop centrifuge (LEFT). Swing bucket rotor in Beckman Coulter Allegra X-15R benchtop centrifuge (RIGHT).

A centrifugation step was also implemented after adding bacteria to the **tissue culture plate** with HaCaT cells in order to increase attachment. The initial method was changed so that the plate was centrifuged at 900xG for 10 minutes (Eppendorf 5430 R, rotor A-2-MTP) at 37 °C after adding bacteria, and then incubated at 37 °C and 5% CO_2 for 50 minutes.

For **FACS samples**, both a swing bucket benchtop centrifuge and ultracentrifugation were tested. Two steps of ultra centrifugation were chosen for the optimized surface shaving experiment, the first with a full SW28 rotor for each sample (6 large tubes), and the last with one tube of resuspended pellets in a SW50.1 rotor (1 small tube). The centrifugations were done at 10000xG.

3.5.4 Culture conditions: used or unused DMEM with 10% FBS

Comparison of *S. haemolyticus* 53-38 growth in secondary cultures (3.2.2.2) of used and unused DMEM with 10% FBS was done. The used DMEM with 10% FBS had been used

overnight as cell culture medium for HaCaT cells. Incubation time and start concentration was equal for all samples.

3.6 Surface shaving of expressed bacterial proteins, optimized experiment

The goal/aim of the optimized surface protein shaving experiment was to increase the bacterial concentration after FACS before inoculating the LPI™ Flow Cell. An alternative approach was also designed in order to ensure sufficient bacterial concentrations.

Four different sets of samples were set up in three parallels and run on the LPI™ Flow Cell by Nanoxis. Sample description in Table 8 below.

Table 8 – Overview of samples for bacterial surface shaving of proteins. Methods in chronological order. Green = performed, orange = not performed.

Handling of samples	**Sample 1-3**	**Sample 4-6**	**Sample 7-9**	**Sample 10-12**
Primary TSB culture	x	x	x	x
Secondary culture in unused cell culture medium DMEM with 10% FBS	x	x	-	x
Secondary culture in used cell culture medium DMEM with10% FBS	-	-	x	-
Washing secondary overnight cultures in PBS	x	x	x	x
Resuspended in unused cell culture medium DMEM with 10% FBS	x	-	-	-
Resuspended in PBS	-	x	x	x
Incubating *S. haemolyticus* with HaCaT cells in tissue culture plates	x	-	-	-
FACS	x	x	-	-
Ultra centrifugation after FACS	x	x	-	-
LPI™ Flow Cell	x	x	x	x

Sample 1-6 / 10-12: Secondary culture (1:200 dilution from primary culture) in **unused** DMEM with 10% FBS (3.2.2) was incubated for 12 hours ± 45 minutes to OD_{600} 0.6-0.7.

Sample 7-9: Secondary culture (1:200 dilution from primary culture) in **used** DMEM with 10% FBS (3.2.2) was incubated for 12 hours ± 45 minutes to OD_{600} 1.6-1.7. Used cell culture medium was obtained from HaCaT culture flasks after one night of cell growth (3.2.3.1).

Secondary cultures (sample 1-12) were washed in a swing bucket centrifuge (Beckman Coulter Allegra X-15R) (3.2.2.2). Samples 1-3 were resuspended in DMEM with 10% FBS

(ready for HaCaT), samples 4-6 were resuspended in PBS (ready for FACS) and samples 7-12 were diluted with PBS to OD_{600} 1.96 (ready for LPI™ Flow Cell).

Two 6 well tissue culture plates with HaCaT cells were used for each sample (sample 1-3) (3.2.3.2). Tissue culture plates with eukaryotic cells were incubated for 22-24 hours before adding bacteria (3.2.4) and preparing samples (3.2.5) prior to cell sorting on FACS (3.2.6).

3.6.1 FACS and ultracentrifugation (samples 1-6)

After sorting on FACS (3.2.6), the samples (1-6) were subsequently centrifuged twice in ultracentrifuge Optima LE-80K with two different rotors (Beckman Coulter).

First ultracentrifugation:

- Rotor SW 28, six large ultracentrifuge tubes (25x89mm, Beckman Coulter).
- 8700 rpm (10000xG) for 30 minutes.
- The pellet was resuspended in excess liquid from FACS (if available) or the supernatant after the first ultracentrifugation and transferred to one small ultracentrifuge tube.

Second ultracentrifugation:

- Rotor SW 50.1, one small ultracentrifuge tube (15x51mm, Beckman Coulter).
- 10300 rpm (10000xG) for 30 minutes.
- Supernatant was removed and resuspended to a volume of 45 μl, which was the volume necessary for Nanoxis to run the Flow cell.

3.6.2 LPI™ Flow cell and proteomics (samples 1-12)

Resuspended pellets from 2 times of ultra centrifugation after FACS were delivered to Nanoxis for proteomics. A TMT tag was used for *S. haemolyticus* samples 1-12 to do a relative quantification study and evaluate up- and downregulation of proteins (3.2.8).

The up- and downregulation was based on the calculated fold change where the average intensity of the TMT tag of the biological replicates of the study group (e.g. HaCaT colonisation prior to surface shaving) was compared to the control group (e.g. no HaCaT colonisation prior to surface shaving).

Prediction of the subcellular localization of proteins was done with PSORTb v.3.0 algorithms (Yu et al., 2010). Functional annotation of proteins was done with the EggNOG v.4.5.1 database; i.e. functional description and categorization of proteins into Clusters of Orthologous Groups of proteins (COG) (Powell et al., 2014). Protein BLAST was done for some of the results via the National Center for Biotechnology Information (NCBI).

4 Results

4.1 Preliminary work

4.1.1 Optimization of growth media for *S. haemolyticus*

Initial experiments with *S. haemolyticus* 57-26 grown in different media showed that TSB was the optimal growth medium based on OD_{600} measurements. The bacterial growth reached stationary phase after ~7-8 hours in TSB and ~16-18 hours in DMEM with 10% FBS and 10% TSB.

To investigate if TSB was a necessary additive to DMEM with 10% FBS for sufficient *S. haemolyticus* growth, an experiment with DMEM with 10% FBS and various concentrations of TSB (0-10%) was performed (Figure 10). OD_{600} of 57-26 and 53-38 in DMEM with 10% FBS and without TSB was similar (Figure 10). OD_{600} of 57-26 and 53-38 after **25** and **24** hours of incubation respectively in DMEM with 10% FBS without TSB (Figure 10) were 1.19 for 53-38 and 1.07 for 57-26. OD_{600} of 57-26 in DMEM with 10% FBS and 5% TSB was 1.8, and in DMEM with 10% FBS and 10% TSB it was 1.7 after **24** hours of growth.

CFU/ml for *S. haemolyticus* 53-38 grown in DMEM with 10% FBS without TSB ranged from 1.6×10^7 after **3 hours and 15 minutes** to 4.8×10^7 after **9 hours**.

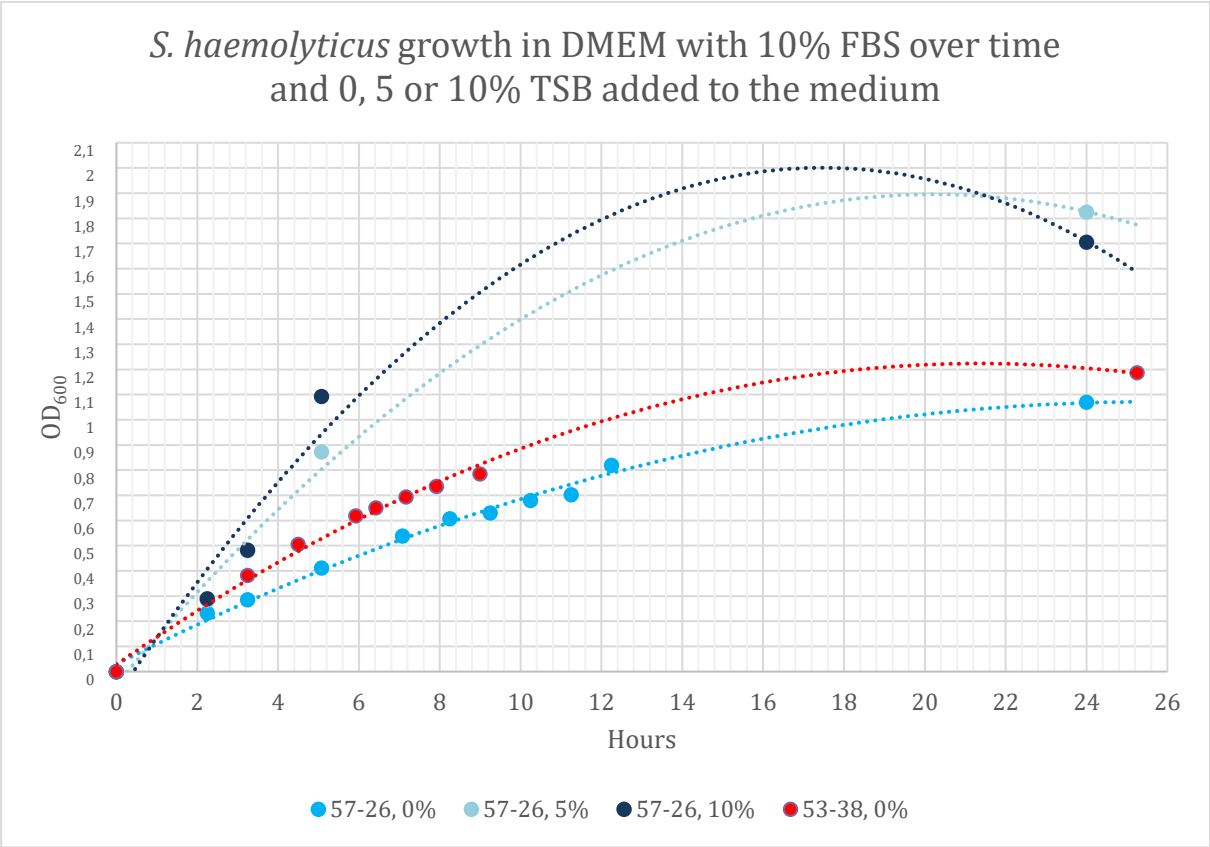


Figure 10 - Comparison of S. haemolyticus growth over 25 hours. Growth of S. haemolyticus 57-26 was done with 0%, 5% or 10% TSB added to cell culture medium, and S. haemolyticus 53-38 without TSB added in DMEM with 10% FBS.

4.1.2 FACS

FACS was tested and settings for sorting bacterial particles were determined before the initial surface protein shaving experiment. The gate for sorting bacteria was set, and was based on size and fluorescence. When determining the concentration of bacterial cells in the FACS test experiments, CFU/ml were 10^6-10^7 for samples before FACS and 10^5-10^6 in sample tubes after sorting on FACS.

4.2 Surface shaving of expressed bacterial proteins, initial experiment

CFU/ml was calculated for samples in the initial surface protein shaving experiment (Table 9). Protein results are described in chapter 4.2.1.

Table 9 - CFU/ml and volumes for different samples and conditions during the initial surface protein experiment. Two parallels were used to calculate SD.

53-38, type of sample	**Secondary culture media**	**Sample no.**	**Volume of resuspended pellet**	**CFU/ml, mean value (SD, σ)**
Resuspended *S. haemolyticus* after centrifugation of secondary culture, added to HaCaT cells	DMEM with 10% FBS	1	-	3.6×10^7 (0.15)
		2	-	5.3×10^7 (0.1)
		3	-	5.4×10^7 (0.4)
Scraped HaCaT and *S. haemolyticus* from tissue culture plates	DMEM with 10% FBS	1	-	1.4×10^7 (0)
		2	-	1.8×10^7 (0.35)
		3	-	2.2×10^7 (0.05)
Resuspended *S. haemolyticus* after centrifugation of secondary culture	TSB	4	-	6.9×10^8 (0.3)
		5	-	8.2×10^8 (0.25)
		6	-	1.0×10^9 (0)
Resuspended *S. haemolyticus* after FACS and centrifugation	TSB	4	100 µl	1.6×10^5 (No parallels)
		5	150 µl	1.1×10^5 (0.05)
		6	150 µl	6.8×10^4 (0.6)
	DMEM with 10% FBS	1	200 µl	1.3×10^5 (7.95)
		2	300 µl	2.1×10^5 (0.05)
		3	250 µl	3.0×10^5 (0.65)

4.2.1 Protein analysis, initial surface shaving experiment

The protein TMT analysis for sample 1-6 was negative, probably caused by a too low concentration of bacterial cells added to the LPI™ Flow Cell.

Four hundred and seven proteins were found when doing a general protein analysis (no TMT analysis) of the TSB overnight cultures (samples 7-9). The number of shared and unique proteins is shown in Figure 11.

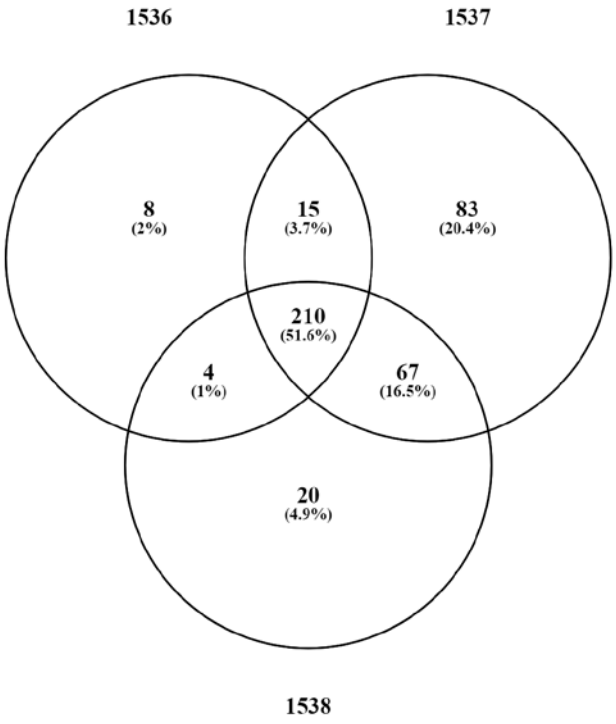


Figure 11 - Venn diagram of shared and unique number of protein identifications for the individual samples of S. haemolyticus searched against the 53-38 genome. 1536, 1537 and 1538 was the ID of the S. haemolyticus parallels (sample 7-9) when performing the protein analysis.

4.3 Optimization of the initial surface shaving experiment

4.3.1 Conditions of the LPI™ Flow Cell

Nanoxis ran a protein analysis with different standards to see what kind that gave least lysis of *S. haemolyticus*. Trypsin 40 μg/ml in PBS was chosen as the standard for the next main experiment.

4.3.2 Survival in PBS after centrifugation

We investigated if *S. haemolyticus* might be killed by prolonged incubation in PBS. This was tested for up to 70 minutes by determining CFU/ml. CFU/ml did not decrease over time. After running FACS and centrifugation, CFU/ml of a *S. haemolyticus* suspension was 8×10^5 after 0 min, 35 min and 70 minutes.

4.3.3 Optimization of centrifugation

Centrifuging bacterial suspensions in a swing bucket benchtop centrifuge (CFU/ml, four parallels, mean $1.5x10^9$, σ=0.4) for 30 minutes increased the concentration (CFU/ml) of the resuspended pellets 5.4 times compared to the fixed angle benchtop centrifuge (CFU/ml, four parallels, mean $2.8x10^8$, σ=0.4). A swing bucket centrifuge was implemented for centrifugation of overnight cultures for the optimized experiments.

A centrifugation step was implemented (900xG for 10 minutes) after *S. haemolyticus* had been transferred to tissue culture plates with HaCaT cells, and before incubation. When comparing samples from centrifuged and non-centrifuged plates, the number of sorted particles on FACS (i.e. bacteria) increased 7.4 times for samples from centrifuged plates (two parallels mean $1.4x10^7$, σ=0.0) compared to non-centrifuged plates (two parallels mean $1.9x10^6$, σ=0.3).

In order to further concentrate the samples, centrifugation in an ultracentrifuge was tested. The concentration (CFU/ml) of the resuspended pellet after using the ultracentrifuge (CFU/ml, four parallels, mean $1.7x10^7$, σ=0.3) was 3.5 times higher than when using the swing bucket benchtop centrifuge (CFU/ml, four parallels, mean $4.9x10^6$, σ=1.0).

Different approaches for ultracentrifugation of samples after FACS were tested. When scraped cells were transferred to the sample tube before running FACS, the volume was 5 ml. During FACS, the volume increased to ~200-250 ml, enough to fill all six tubes in the largest ultracentrifuge rotor (SW28). After one round of centrifugation, the sample volume left in the tube after removal of the supernatant was too large (CFU/ml resuspended pellet, two parallels, mean $4.7x10^6$, σ=0.3). An extra centrifugation step was then implemented (Table 10) to further concentrate the sample. The pellets were resuspended in the left over liquid in each of the six large tubes, pooled and added to one small ultra centrifuge tube (rotor SW50.1), increasing the sample concentration after ultracentrifugation 6 times (two centrifugation steps) compared to the previous experiment (one centrifugation step).

Table 10 – Optimization of ultracentrifugation of S. haemolyticus 53-38. Swing bucket centrifuge, Allegra X-15R was used to wash overnight cultures. The tissue culture plates were centrifuged at 900xG after adding bacteria to the HaCaT cells. One round of ultracentrifugation was done with the largest rotor, SW28 (six tubes, 250 µl), and a second round of centrifugation was done with the smallest rotor, SW50.1, with the resuspended pellets from the first round of ultra centrifugation in one ultra centrifuge tube. 50 µl of sample material was left in the tube after removing the supernatant and resuspending the pellet in the remaining supernatant after the second round of ultracentrifugation. Two parallels were used to calculate SD.

Sample **53-38**	**CFU/ml, mean value (SD, σ)**
Resuspended *S. haemolyticus* after centrifugation of secondary culture, added to HaCaT cells	9.4×10^7 (0.6)
Scraped HaCaT and *S. haemolyticus* from 6 well tissue culture plates, plate centrifuged 900xG for 10 minutes before 50 minutes of incubation	6.6×10^7 (0.2)
After FACS	3.8×10^5 (0.2)
Resuspended *S. haemolyticus* after FACS and two rounds of ultracentrifugation Rotor first centrifugation: SW28 (large ultracentrifuge tubes) Rotor second centrifugation: SW50.1 (small ultracentrifuge tube) 10000xG, 30 minutes for each centrifugation	2.8×10^7 (0.25)

4.3.4 Culture conditions: used or unused DMEM with 10% FBS

When comparing *S. haemolyticus* growth in used and unused DMEM with 10% FBS, the concentration (CFU/ml) was 16.7 times higher for *S. haemolyticus* grown in used medium compared to unused medium (Table 11).

Table 11 – Comparison of growth of S. haemolyticus 53-38 secondary overnight cultures in used and unused DMEM with 10% FBS. Two parallels were used to calculate SD.

Sample 53-38	**OD_{600} after overnight incubation**	**CFU/ml, mean value (SD, σ)**
Used DMEM with 10% FBS	1.6	7.7×10^7 (0.1)
Used DMEM with 10% FBS	1.6	3.5×10^7 (0.2)
Unused DMEM with 10% FBS	0.5	3.3×10^6 (0)
Unused DMEM with 10% FBS	0.5	3.4×10^6 (0.1)

4.4 Surface shaving of expressed bacterial proteins, optimized experiment

CFU/ml was calculated for samples in the optimized surface protein shaving experiment (Table 12). Protein results are described in chapter 4.4.1.

Table 12 - CFU/ml, OD_{600} and volumes of the different samples and conditions during the optimized surface protein shaving experiment. NA = Not Available (e.g. not enough sample to do dilutions). A more detailed table can be viewed in the appendix. Two parallels were used to calculate SD.

53-38, type of sample	**Sample no.**	**OD_{600}**	**Volume of resuspended pellet (45 µl needed)**	**CFU/ml, mean value (SD, σ)**
Overnight culture in DMEM with 10%FBS	1	0.582	.	NA (NA)
	2	0.540	.	1.2×10^7 (0.1)
	3	0.693	.	1.8×10^7 (0)
	4	0.696	.	2.1×10^7 (0.1)
	5	0.684	.	1.8×10^7 (0.15)
	6	0.693	.	2.1×10^7 (0)
	10	0.696	.	1.5×10^7 (0.25)
	11	0.740	.	2.2×10^7 (0.15)
	12	0.692	.	2.6×10^7 (0.2)
Overnight culture in used DMEM with 10% FBS	7	1.673	.	3.5×10^8 (0.45)
	8	1.671	.	4.5×10^8 (0.2)
	9	1.649	.	3.7×10^8 (0.15)
Resuspended *S. haemolyticus* after centrifugation of secondary culture, added to HaCaT cells	2	.	.	1.7×10^7 (0.25)
Scraped HaCaT and *S. haemolyticus* from tissue culture plates	1	.	.	1.8×10^7 (0.45)
	2	.	.	2.7×10^7 (0.1)
	3	.	.	1.2×10^7 (0) (Less sample material due to an centrifugation error)
FACS liquid	4	.	.	2.0×10^5 (0.05)
	6	.	.	2.0×10^5 (No parallels)
Supernatant after FACS and ultra centrifugation	1	.	.	2×10^1 (2)
	2	.	.	4.6×10^2 (1.4)
	5	.	.	6×10^1 (2)
	6	.	.	0 (0)
Resuspended *S. haemolyticus* after	1	.	40 µl	NA (NA)
	2	.	45 µl	NA (NA)
	3	.	30 µl + 15 µl supernatant	NA (NA)

FACS and ultra centrifugation	4	.	35 µl + 15 µl supernatant	NA (NA)
	5	.	30 µl + 15 µl supernatant	NA (NA)
	6	.	55 µl (45 µl to flow cell analysis by Nanoxis and 10 µl for dilution and CFU count)	7.8 x 10^6 (No parallels)
Wash liquid from LPI™ Flow Cell	1	.	.	8 x 10^3 (No parallels)
	2	.	.	3 x 10^4 (0)
	3	.	.	1.2 x 10^4 (0.2)
	4	.	.	3.1 x 10^3 (0.7)
	5	.	.	1.5 x 10^3 (0.4)
	6	.	.	2.1 x 10^3 (0.1)

4.4.1 Protein analysis, optimized surface shaving experiment

Protein results from the optimized surface shaving experiment were received close to master thesis deadline. A summary of some potentially important findings are described in the next chapters.

4.4.1.1 HaCaT colonisation experiment

Surface shaving of *S. haemolyticus* after HaCaT colonisation resulted in the identification of 319 proteins by MS. Eighteen (5.6%) of the proteins were strongly upregulated (Table 13), 41 (12.9%) slightly upregulated, 14 (4.4%) strongly downregulated (Table 13) and 62 (19.4%) slightly downregulated after HaCaT co-colonisation, compared to samples treated equally, except for the HaCaT colonisation step (control group). The rest of the proteins did not show any up- or downregulation.

The up- and downregulation of the HaCaT model is determined and sorted by the fold change, the average intensity of the TMT tag of the biological replicates of the HaCaT colonisation model compared to the control group. The up- and downregulated proteins found when using the alternative approach, i.e. incubating *S. haemolyticus* in used cell culture medium vs. unused medium, are also listed in the tables (Table 13 and 14).

The calculated fold change of detected proteins in the HaCaT colonisation model was 1.53-3.82 for strongly upregulated proteins, 1.20-1.49 for slightly upregulated proteins, 0.67-0.83 for slightly downregulated proteins and 0.49-0.66 for strongly downregulated

proteins. The fold change for the rest of the proteins was in-between the up- or downregulated values.

Twelve cell wall and eight extracellular proteins were found among the 319 proteins when predicting subcellular localization with PSORTb (Table 14, Figure 13). Two of the cell wall and two of the extracellular proteins were strongly upregulated. Fifty cytoplasmic membrane and 211 cytoplasmic proteins were predicted, and 38 had unknown function.

Six adhesion and virulence proteins were detected among the eighteen strongly upregulated proteins; three proteins with YSIRK/LPXTG motifs, possibly involved in attachment to host; one reported important for attachment to nares of rats; AtlE, possibly involved in attachment to host; one heme oxygenase using host heme as an iron source. Among the slightly upregulated proteins, a Tir protein was found, possibly increasing bacterial survival in the host (PSORTb: cytoplasmic, COG: S, EggNOG: tir protein, pBLAST: "Multispecies: TIR domain-containing protein (Staphylococcus)"). Lytic motifs (LysM or CHAP) could be found among the slightly upregulated extracellular proteins (Table 14).

The 319 proteins found constitute 12.6% of the total CDS (predicted proteins) in the genome of the strain.

Table 13 –18 proteins were strongly upregulated and 14 were strongly downregulated during S. haemolyticus colonisation of HaCaT cells, all listed in the table below. Dark green = Strongly upregulated, light green = slightly upregulated, dark red = strongly downregulated, light red = slightly downregulated, white = no up- or downregulation, grey = protein not found. Cluster of Orthologous Groups (COG) descriptions can be found in Figure 14.

Accession (Contig-gene)	**Up- and down-regulation based on fold change**		**PSORTb: subcellular localization**	**COG**	**EggNOG: Functional annotation**	**NCBI: Protein BLAST**
	HaCaT model	**Used medium**				
18-22	↑↑	Not found	Cytoplasmic	C	dihydrolipoyl dehydrogenase	
52 -1	↑↑	↑↑	Cell wall	M	Cell surface-associated protein implicated in **virulence** by promoting bacterial **attachment** to both alpha- and beta-chains of human fibrinogen and inducing the formation of bacterial clumps	Hypothetical protein/**YSIRK** signal domain/**LPXTG** anchor domain surface protein (*S. haemolyticus*)

38 -9	↑↑	↑	Cytoplasmic	P	Allows bacterial pathogens to **use the host heme as an iron source**. Catalyzes the oxidative degradation of the heme macrocyclic porphyrin ring to the oxo-bilirubin chromophore staphylobilin (a mixture of the linear tetrapyrroles 5-oxo-delta- bilirubin and 15-oxo-beta-bilirubin) in the presence of a suitable electron donor such as ascorbate or NADPH—cytochrome P450 reductase, with subsequent release of free iron (By similarity)	**Heme oxygenase** (*S. haemolyticus)*
18 -21	↑↑	↓↓	Cytoplasmic	C	branched-chain alpha-keto acid dehydrogenase subunit E2	
81-1	↑↑	↑	Cell wall	S	**YSIRK adhesion** protein *S.haemolyticus*	**Adhesin**/Hypothetical protein/**YSIRK** signal domain/**LPXTG** anchor domain surface protein (*S. haemolyticus*)
19-39	↑↑	↑	Cytoplasmic	H	Catalyzes the formation of S-adenosylmethionine from methionine and ATP	
3-1	↑↑	↑	Unknown	S	UPF0337 protein,CsbD family protein, general stress protein	
17-54	↑↑	↓	Extracellular	M	**transglycosylase SceD**, important for **colonisation of nares** of cotton rats (J. Bacteriol. October 2007 vol. 189 no. 20 7316-7325) Is a *S. aureus* surface antigen	
12-61	↑↑	–	Cytoplasmic	S	Dehydrogenase, Enoyl-[acyl-carrier-protein] reductase (NADH), FAbI	
10-53	↑↑	–	Cytoplasmic	F	uridine monophosphokinase	
66-1	↑↑	↑↑	Unknown	S	**YSIRK/LPXTG** protein	Collagen-binding protein/Hypothetical protein/**YSIRK** signal domain/**LPXTG** anchor domain surface protein (*S. haemolyticus*)
32-12	↑↑	↑↑	Extracellular	G	**mannosyl-glycoprotein endo-beta-N-acetylglucosamidase, lytD, CwlA, AtlE surface antigen**?	**mannosyl-glycoprotein endo-beta-N-acetylglucosamidase** (*S. haemolyticus*)
23-17	↑↑	–	Cytoplasmic	J	This is one of the proteins that binds to the 5S RNA in the ribosome where it forms part of the central protuberance (By similarity)	
14-69	↑↑	–	Cytoplasmic	C	Key enzyme in the regulation of glycerol uptake and metabolism (By similarity)	
21-41	↑↑	–	Cytoplasmic	E	Dehydrogenase	
1-133	↑↑	↓	Cytoplasmic	J	phenylalanyl-tRNA synthetase (beta subunit)	
17-38	↑↑	↓	Cytoplasmic	F	Catalyzes the conversion of uracil and 5-phospho-alpha- D-ribose 1-diphosphate (PRPP) to UMP and diphosphate (By similarity)	
40-22	↑↑	↑	Cytoplasmic	C	aldo keto reductase	

8 -21	↓↓	–	Cytoplasmic	V	Catalyzes the incorporation of amino acid(s) into the interchain peptide bridge of peptidoglycan, using aminoacyl-tRNA as amino acid donor (By similarity)	
2-119	↓↓	↓	Unknown	G	fructose-bisphosphate aldolase	
11-1	↓↓	–	Extracellular	P	Destroys radicals which are normally produced within the cells and which are toxic to biological systems (By similarity)	
3-9	↓↓	↓	Cytoplasmic	E	The glycine cleavage system catalyses the degradation of glycine. The H protein shuttles the methylamine group of glycine from the P protein to the T protein (By similarity)	
17 -48	↓↓	↑	Cytoplasmic	C	Produces ATP from ADP in the presence of a proton gradient across the membrane (By similarity)	
8-14	↓↓	↓	Cytoplasmic Membrane	P	phosphate ABC transporter (Permease)	
3 -3	↓↓	↑↑	Cytoplasmic Membrane	P	Lipoprotein	
15-65	↓↓	–	Cytoplasmic	K	DNA-dependent RNA polymerase catalyses the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates (By similarity)	
8 -19	↓↓	↑↑	Cytoplasmic Membrane	P	Periplasmic binding protein	
4 -35	↓↓	–	Cytoplasmic Membrane	E, P	ABC transporter	
27- 5	↓↓	↑↑	Cytoplasmic	O	Participates actively in the response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins, in association with DnaK and GrpE. It is the nucleotide exchange factor for DnaK and may function as a thermosensor. Unfolded proteins bind initially to DnaJ	
35-10	↓↓	–	Cytoplasmic	J	cysteinyl-tRNA synthetase	
3-137	↓↓	↑	Cytoplasmic	J	ribosomal subunit Interface protein	
3-111	↓↓	↓	Cytoplasmic	G	Triose-phosphate isomerase	
28-18	↓↓	↑	Cytoplasmic	E	Glycine cleavage system H protein	

Table 14 – All cell wall (12) and extracellular (8) proteins found in the HaCaT colonisation experiment when predicting subcellular localization with PSORTb v.3.0. All of the proteins, except two, were also found when doing the used cell culture medium experiment. COG descriptions can be found in Figure 14.

Accession (Contig-gene)	**Up- and down-regulation**		**PSORTb: subcellular localization**	**COG**	**EggNOG: Functional annotation**	**Protein BLAST**
	HaCaT model	**Used medium**				
52-1	↑↑	↑↑	Cell wall	M	Cell surface-associated protein implicated in **virulence** by promoting bacterial attachment to both alpha- and beta-chains of human fibrinogen and inducing the formation of bacterial clumps	
81-1	↑↑	↑	Cell wall	S	**YSIRK** adhesion protein *S. haemolyticus*	
18-41	–	↓↓	Cell wall	Q	alkaline phosphatase	

2-27	–	↓↓	Cell wall	Not found	Not found	hypothetical protein (*S. haemolyticus*)
11-71	–	↑	Cell wall	M	elastin-binding protein ebpS	
58-1	–	↑↑	Cell wall	O	Inherit from COG: peptidase (S8 and S53, subtilisin, kexin, sedolisin	
2-113	–	↓	Cell wall	C	malate dehydrogenase (quinone)	
2-22	–	–	Cell wall	E	Substrate-binding region of abc-type glycine betaine transport system	
6-83	–	–	Cell wall	O	Heat shock protein (HSP20)	
4-50	–	Not found	Cell wall	U	Signal peptidase i	
4-33	↓	–	Cell wall	E	Extracellular solute-binding protein, family 5	
22-38	↓	–	Cell wall	Not found	Not found	**LPXTG** cell wall anchor domain-containing protein (*S. haemolyticus*)
17-54	↑↑	↓	Extracellular	M	transglycosylase SceD, important for **colonisation of nares** of cotton rats (J. Bacteriol. October 2007 vol. 189 no. 20 7316-7325) Is a S.aureus surface antigen	
32-12	↑↑	↑↑	Extracellular	G	**mannosyl-glycoprotein endo-beta-N-acetylglucosamidase, lytD, CwlA, AtlE surface antigen**?	**mannosyl-glycoprotein endo-beta-N-acetylglucosamidase** (*S. haemolyticus*)
21-28	↑	↑↑	Extracellular	S	**LysM**	
15-1	↑	↓↓	Extracellular	M	**CHAP** domain containing protein, **surface antigen**	
2-33	–	↑↑	Extracellular	Not found	Not found	hypothetical protein (*S. haemolyticus*)
1-171	–	Not found	Extracellular	S	(LipO) protein	
36-6	↓	–	Extracellular	Not found	Not found	hypothetical protein (*S. haemolyticus*)
11-1	↓↓	–	Extracellular	P	Destroys radicals which are normally produced within the cells and which are toxic to biological systems (By similarity)	

4.4.1.2 Used vs. unused DMEM with 10% FBS experiment

Using the alternative approach culturing *S. haemolyticus* in used vs. unused DMEM with 10% FBS, resulted in the identification of in total 794 proteins by MS. One hundred and six (13.4%) proteins were strongly upregulated, 117 (14.7%) proteins slightly upregulated, 77 (9.7%) proteins strongly downregulated and 159 (20.0%) proteins slightly downregulated during incubation in used cell culture medium compared to the profiles of bacteria grown in unused medium (control group). The rest of the proteins did not show any up- or downregulation. The top ten strongly up- and downregulated

proteins are listed in Table 15. The up- and downregulation of proteins identified when using the HaCaT colonisation model are also listed.

The calculated fold change of detected proteins in the used vs. unused cell culture medium model was 1.50-22.77 for strongly upregulated proteins, 1.20-1.50 for slightly upregulated proteins, 0.67-0.83 for slightly downregulated proteins and 0.34-0.67 for strongly downregulated proteins. The fold change for the rest of the proteins were in-between the up- or downregulated values.

Eighteen cell wall and sixteen extracellular proteins were found among the 794 proteins when predicting subcellular localization with PSORTb (Table 16, Figure 13). The ones that are also found in the HaCaT colonisation are listed in Table 14. Four cell wall and six extracellular proteins were strongly upregulated .

An AtlE, possibly involved in attachment to host was found among the top ten strongly upregulated proteins (Table 15). LysM was found among the strongly upregulated extracellular proteins (Table 14). A nuclease (strongly upregulated), not previously described in the HaCaT colonisation model was found among the extracellular proteins in the used medium model (Table 16).

The 794 proteins found constitute 31.3% of the total CDS in the genome of the strain.

Table 15 –106 proteins were strong upregulated and 77 were strong downregulated in the experiment with used cell culture medium. The top 10 up- and downregulated proteins are listed in the table below. COG descriptions can be found in Figure 14.

Accession (Contig-gene)	**Up- and downregulation**		**PSORTb: subcellular localization**	**COG**	**EggNOG: Functional annotation**	**Protein BLAST**
	Used medium	**HaCaT model**				
1-134	↑↑	Not found	Cytoplasmic	J	phenylalanyl-tRNA synthetase (alpha subunit)	
2-33	↑↑	–	Extracellular	Not found	Not found	hypothetical protein (*S. haemolyticus*)
32-12	↑↑	↑↑	Extracellular	G	**mannosyl-glycoprotein endo-beta-N-acetylglucosamidase, lytD, CwlA, AtlE surface antigen**?	**mannosyl-glycoprotein endo-beta-N-acetylglucosamidase** (S. *haemolyticus*)
2-35	↑↑	Not found	Extracellular	Not found	Not found	hypothetical protein (*Staphylococcus*)

8-41	↑↑	–	Cytoplasmic	L	DNA topoisomerase IV, subunit A	
9-11	↑↑	Not found	Unknown	Not found	Not found	V8-like Glu-specific endopeptidase / serine protease (*S. haemolyticus*)
11-21	↑↑	–	Unknown	P	rhodanese family	
12-19	↑↑	Not found	Unknown	P	(LipO) protein	
10-2	↑↑	Not found	Cytoplasmic	H	Catalyzes the NADPH-dependent reduction of glutamyl- tRNA(Glu) to glutamate 1-semialdehyde (GSA) (By similarity)	
2-56	↑↑	Not found	Cytoplasmic membrane	M	Capsular exopolysaccharide family	
35-21	↓↓	Not found	Cytoplasmic	H	Involved in the production of pyridoxal phosphate, probably by incorporating ammonia into the pyridine ring (By similarity)	
1-170	↓↓	–	Cytoplasmic	C	(E1 component), alpha subunit	
42-14	↓↓	Not found	Cytoplasmic	H	glutamate-1-semialdehyde aminotransferase	
20-9	↓↓	Not found	Cytoplasmic	E	Glutamate synthase	
8-15	↓↓	–	Cytoplasmic Membrane	P	Part of the ABC transporter complex PstSACB involved in phosphate import. Responsible for energy coupling to the transport system (By similarity)	
5-65	↓↓	Not found	Cytoplasmic	M	teichoic acid biosynthesis protein X	
16-24	↓↓	–	Cytoplasmic	C	d-lactate dehydrogenase	
8-31	↓↓	Not found	Cytoplasmic	E	m42 family	
6-26	↓↓	Not found	Cytoplasmic	C	Dehydrogenase	
3-113	↓↓	↓	Cytoplasmic	G	Glyceraldehyde-3-phosphate dehydrogenase	

Table 16 - Cellwall and extracellular proteins found in the used cell culture medium model, but not in the HaCaT colonisation experiment. COG descriptions can be found in Figure 14.

Accession (Contig-gene)	**Up- and down-regulation**	**PSORTb: subcellular localization**	**COG**	**EggNOG: Functional annotation**	**Protein BLAST**
20-16	↑↑	Cell wall	M	**LysM**	
84-1	↑↑	Cell wall	S	surface protein	
1-105	–	Cell wall	M	Cell division protein that may be involved in stabilizing or promoting the assembly of the division complex (By similarity)	

41-9	–	Cell wall	S	surface protein	
33-5	–	Cell wall	F	5'-nucleotidase	
56-1	–	Cell wall	S	surface protein	
12-28	↓	Cell wall	C	malate dehydrogenase (quinone)	
2-35	↑↑	Extracellular	Not found	Not found	hypothetical protein (*Staphylococcus*)
1-61	↑↑	Extracellular	L	**nuclease**	
9-55	↑↑	Extracellular	S	Triacylglycerol lipase	
63-4	↑	Extracellular	Not found	Not found	hypothetical protein (*S. haemolyticus*)
4-79	–	Extracellular	S	Protein of unknown function (DUF1462)	
80-2	–	Extracellular	G	**mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase, AtlE**	
2-30	–	Extracellular	N, U	**n-acetylmuramoyl-l-alanine amidase**	
11-24	–	Extracellular	Not found	Not found	hypothetical protein (*S. haemolyticus*)
2-91	↓↓	Extracellular	N, U	domain protein	

4.4.1.3 Comparison of experiments; HaCaT colonisation vs. used DMEM with 10% FBS

319 proteins were identified in the HaCaT colonisation experiment, whereas 794 proteins were identified in the used cell culture medium. All of the strong (18) and slightly (41) upregulated proteins in the HaCaT colonisation model were compared to the up- and downregulation of the same proteins in the used cell culture model (Figure 12).

Prediction of subcellular localization of proteins with PSORTb v.3.0 was done (Figure 13), and COG groups from EggNOG v4.5.1database were determined (Figure 14).

When predicting subcellular localization with PSORTb of the total CDS of the strain (2537) 1.9% extracellular, 1.5% cell wall, 25.8% cytoplasmic membrane, 50.8% cytoplasmic and 20.1% unknown proteins were found.

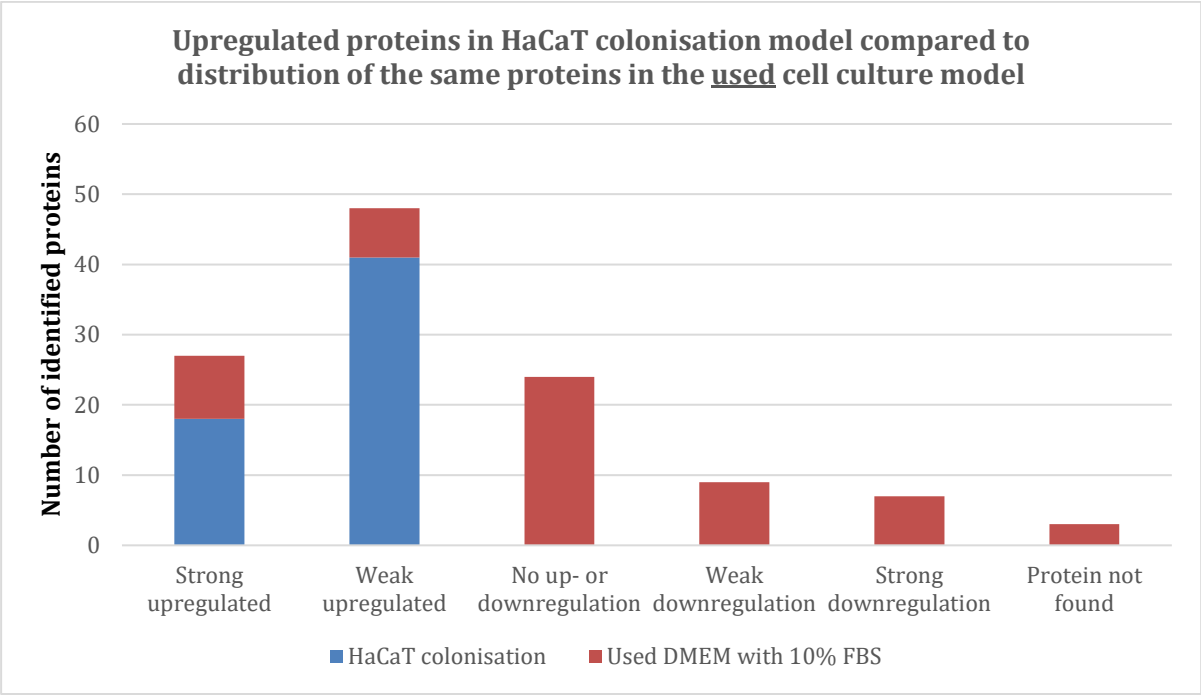


Figure 12 – All upregulated proteins, 18 strong and 41 weak, in HaCaT colonisation experiment compared to the distribution of up- and downregulation of the same proteins in the used DMEM with 10% FBS experiment.

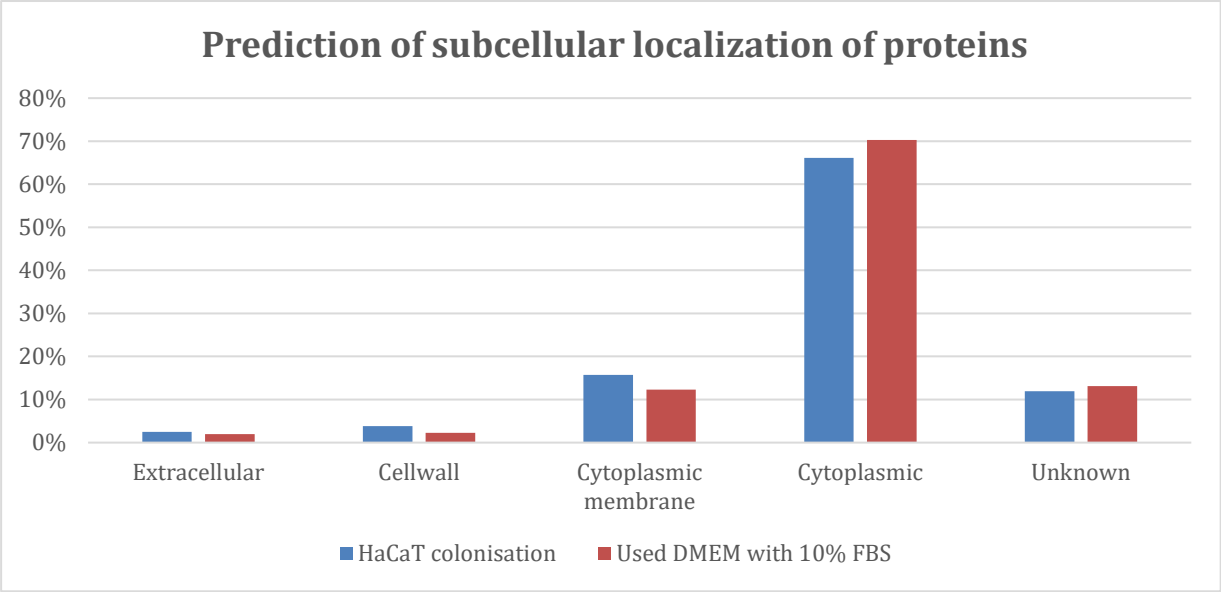


Figure 13 – Prediction of subcellular localization of proteins with PSORTb v3.0. The localization is given as extracellular, cell wall, cytoplasmic membrane, cytoplasmic or unknown. The percentage of the amount in each group is calculated from the total number of proteins. 319 proteins were found in the HaCaT colonisation experiment, and 794 proteins in the used cell culture medium experiment. 8 extracellular (2.5%) and 12 cell wall (3.8%) proteins were found in the HaCaT colonisation experiment, whereas 16 (2.0%) extracellular and 18 (2.3%) cell wall proteins where found in the used cell culture medium experiment.

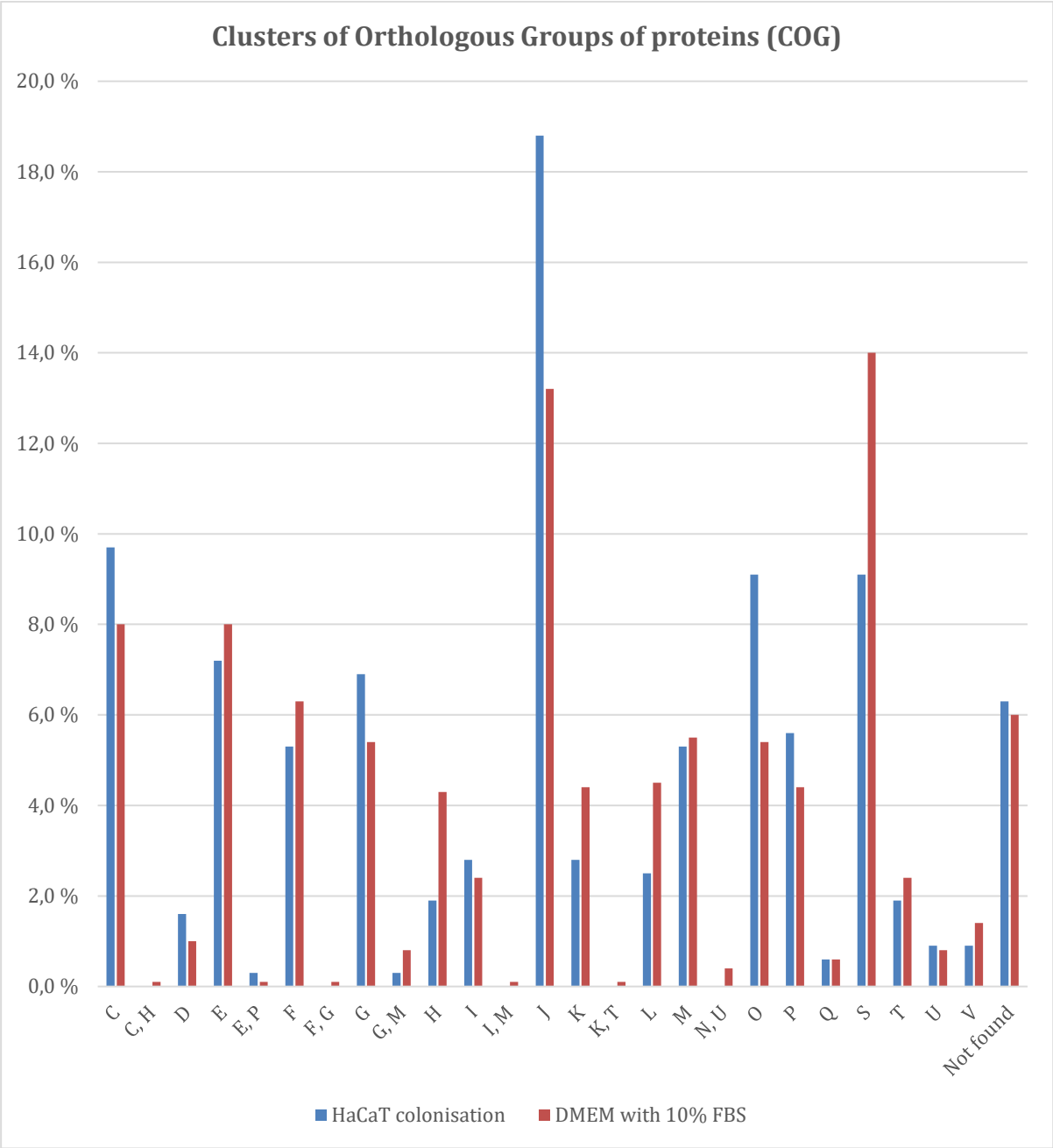


*Figure 14 - Comparison of distribution in COG from EggNOG v4.5.1 between HaCaT colonisation experiment and experiment with <u>used</u> cell culture medium. The percentage of the amount in each group is calculated from the total number of proteins. 319 proteins were found in the HaCaT colonisation experiment, and 794 proteins in the <u>used</u> cell culture medium experiment. COG one letter code descriptions: INFORMATION STORAGE AND PROCESSING: **[J]** Translation, ribosomal structure and biogenesis; **[K]** Transcription; **[L]** Replication, recombination and repair; CELLULAR PROCESSES AND SIGNALING: **[D]** Cell cycle control, cell division, chromosome partitioning; **[V]** Defence mechanisms**;** **[T]** Signal transduction mechanisms**;** **[M]** Cell wall/membrane/envelope biogenesis; **[N]** Cell motility; **[U]** Intracellular trafficking, secretion, and vesicular transport; **[O]** Posttranslational modification, protein turnover, chaperones; METABOLISM: **[C]** Energy production and conversion; **[G]** Carbohydrate transport and metabolism; **[E]** Amino acid transport and metabolism; **[F]** Nucleotide transport and metabolism; **[H]** Coenzyme transport and metabolism; **[I]** Lipid transport and metabolism; **[P]** Inorganic ion transport and metabolism; **[Q]** Secondary metabolites biosynthesis, transport and catabolism; POORLY CHARACTERIZED; **[S]** Function unknown.*

5 Discussion

This is to our knowledge the first described experiment for surface shaving of staphylococcal proteins after direct contact with eukaryotic cells. Gaining information about surface exposed proteins can be important for the discovery and design of novel vaccines and to better understand host-pathogen interactions.

S. haemolyticus is an important opportunistic pathogen in nosocomial FBRIs (Becker et al., 2014, Cavanagh et al., 2014, Czekaj et al., 2015), but published information about virulence factors is scarce compared to literature published on other staphylococcal species. The aim of this thesis was to develop a method for identification of expressed surface proteins of *S. haemolyticus* when colonisation human keratinocytes.

5.1 Study design

S. haemolyticus and HaCaT cells were grown together and separated with FACS before concentrating the samples in a centrifugation step and using the LPI™ Flow Cell technology to do surface protein shaving of the bacteria.

A *S. haemolyticus* isolated from a clinical wound was chosen as this strain showed high levels of adhesion to human cells in previous experiments (unpublished results). Only adherent bacterial cells were investigated as free floating bacteria would be washed away. Adhesion is a critical first step prior to invasion and/or secretion of toxins (Letourneau et al., 2011).

When deciding the type of human cells to work with, it was important to choose cells from areas where *S. haemolyticus* is normally found. HaCaT cells from a human keratinocyte cell line (Boukamp et al., 1988) were chosen for the experiment since *S. haemolyticus* constitute a large proportion of the skin microbiota of humans (Becker et al., 2014, Grice and Segre, 2011, Cavanagh et al., 2016).

We found that *S. haemolyticus* had excellent growth in both used and unused cell culture medium DMEM with 10% FBS, even though the growth was slower than in conventional TSB medium. When *S. haemolyticus* was grown in DMEM with 10% FBS, changing of

media was avoided when the bacteria were added to HaCaT cells. Avoiding a change in media can/will potentially influence the protein expression.

5.2 Optimization of sample preparation

After the initial surface shaving experiment, no proteins were identified using the TMT analysis due to a too low concentration of bacterial cells added to the LPI™ Flow Cell. It was only possible to retrieve results from the general protein analysis with overnight cultures of bacteria grown in TSB. Four hundred and seven proteins were identified and 210 proteins were shared between the three biological replicates. The reason can be due to differences in lysis. The protein analysis was done on over night cultures that had reached stationary phase, and the bacterial solution was not as homogenous as for samples run in the HaCaT colonisation model. After remaining at high density in stationary phase for a period of time after exponential-phase growth, bacterial cells enter death phase (Finkel, 2006).

Optimization of the method was done to increase both the number of bacteria adhering to HaCaT and retrieval of bacteria after FACS before the surface shaving experiment was repeated. Steps that were optimized lead to a 133 times increase of bacteria retrieved after FACS.

The most important change between the initial and the optimized method were the centrifugations. A centrifugation step was implemented when incubating bacteria and human cells, increasing the concentration (CFU/ml) 7.4 times for samples from centrifuged plates compared to non-centrifuged plates.

In the initial surface shaving experiment, a fixed angle centrifuge was used both for centrifugation of overnight cultures and pelleting of samples after FACS. As the pellets after centrifugation of FACS samples were invisible, it was important to choose a centrifugation method where we could locate the pellet, and in a swing bucket centrifuge, it would be located in the bottom of the tube.

When performing the optimized surface shaving experiment, CFU/ml was only determined for one of the six samples after two times of ultracentrifugation. For the other samples, sufficient volumes to perform dilutions and plating could not be

retrieved as the total sample was added to the LPI™ Flow Cell. The CFU/ml of the single sample plated were 37 times higher than in the initial experiment. The CFU/ml was maybe not representative for all the samples, as repeated centrifugation putatively would lead to loss of some of the sample material. This is reflected by the difference observed when comparing the results from the initial optimization and the actual experiment.

The trypsin standard used in the optimized surface shaving experiment was also changed, as the optimized trypsin concentration gave less lysis of bacterial cells than the concentration used in the initial experiment.

5.3 Protein results from optimized surface shaving experiment

In silico analysis of the complete genome of bacterial genomes predicts surface-associated proteins to constitute between 30%-40% of all bacterial proteins (Rodriguez-Ortega et al., 2006). When predicting subcellular localization with PSORTb of the total CDS of the strain (2537) 1.9% extracellular, 1.5% cell wall, 25.8% cytoplasmic membrane, 50.8% cytoplasmic and 20.1% unknown proteins were found.

319 proteins were identified when performing protein surface shaving of *S. haemolyticus* 53-38 in the optimized experiment. The same proteins were found both for bacteria incubated with HaCaT and not incubated with HaCaT prior to FACS and addition to the LPI™ Flow Cell. Eighteen proteins were strongly upregulated (fold change 1.53-3.82) during HaCaT colonisation, among them six adhesion and/or virulence proteins: three proteins with YSIRK/LPXTG motifs, possibly involved in attachment to host; one reported important for attachment to nares of rats; one Atl protein, possibly involved in attachment to host and one heme oxygenase using host heme as an iron source. All of the proteins are discussed below.

Proteins possibly involved in adhesion to the host were identified among the strongly upregulated proteins. Both YSIRK and LPXTG motifs were found, three in total. The YSIRK family is a Gram-positive signal peptide, and the secreted protein is essential for surface protein anchoring and the peptidoglycan envelope (DeDent et al., 2008). The LPXTG motif anchor CWA proteins to the cell wall (Foster et al., 2014). One of the upregulated proteins is possibly involved in bacterial attachment of human fibrinogen

(52-1), and one in binding to collagen (66-1). Fibrinogen is a blood plasma protein, and collagen is found in connective tissues. SdrG in *S. epidermidis* is an example of a LPXTG containing protein that binds to fibrinogen (Herman et al., 2014), and SdrF of *S. epidermidis* can bind to collagen (Di Poto et al., 2015). *Sdr* genes are also described in *S. haemolyticus* (Takeuchi et al., 2005). A SceD protein was found among the strongly upregulated proteins. Lytic transglycosylase SceD from *S. aureus* is reported being important for colonisation of nares of rats, and the protein is expressed during infection (Stapleton et al., 2007). SceD is suggested as a possible vaccine candidate for *S. aureus* (Stapleton et al., 2007).

Autolysin Atl is a bifunctional protein of staphylococci (Becker et al., 2014), and was found among the strongly upregulated proteins. Autolysin has an enzymatic function with hydrolysis of the cell wall peptidoglycan of the bacteria, leading to release of eDNA (Becker et al., 2014). The Atl protein has an amidase and endo-beta-N-acetylglucosaminidase (GL) domains, where the GL domains of *S. aureus* Atl plays a role in binding DNA (Grilo et al., 2014). Endo-beta-N-acetylglucosaminidase is also described to impair the immune response of mice and prevent the response of human lymphocytes (Valisena et al., 1991). Repeating structures R1ab-R2ab of staphylococcal Atl is described to interact with human thrombospondin 1 and vitronectin (Kohler et al., 2014). N-acetylmuramoyl-l-alanine amidase from e.g. autolysins has been suggested as a biomarker for sepsis (Pinheiro da Silva et al., 2016).

A heme oxygenase was found among the strongly upregulated proteins. Heme oxygenases can cleave the porphyrin heme ring and release iron as a nutrient source. The heme oxygenases IsdI and IsdG of *S. aureus* are only expressed under low iron conditions (Lojek et al., 2018). Heme oxygenases are also described in *S. lugdunensis* (Haley et al., 2011).

Among the identified extracellular proteins that were slightly upregulated, Lysin Motif (LysM) and CHAP domain containing protein were found. LysM can attach proteins to the cell surface in a non-covalent manner to peptidoglycan (Buist et al., 2008). LysM domain-containing proteins are virulence factors of human bacterial pathogens, and are for instance described for *S. aureus* (Buist et al., 2008). The CHAP (cysteine, histidine-

dependent amidohydrolases/peptidases) domain is shown to have lytic activities (Horgan et al., 2009).

A TIR domain-containing protein was found among the slightly upregulated proteins. TIR-containing genes have been identified in several bacterial species, and *tirS* can increase *S. aureus* survival in the host (Askarian et al., 2014).

Subcellular localization of proteins were predicted with the PSORTb algorithms, where 12 cell wall (3.8%), 8 extracellular (2.5%), 50 cytoplasmic membrane (15.7%), 211 cytoplasmic (66.1%) and 38 unknown (11.9%) proteins were found among the 319 proteins. Predicted cytoplasmic proteins are normally found when performing surface shaving techniques (Olaya-Abril et al., 2014, Solis and Cordwell, 2016), and their presence is discussed more detailed in the next chapter (5.4).

The PSORTb prediction of subcellular localization of proteins were similar between the two different approaches; The HaCaT colonisation model and used cell culture medium model, but a higher degree of predicted cytoplasmic proteins were found in the used cell culture medium model, possibly indicating a higher degree of cellular lysis.

The genome of *S. haemolyticus* 53-38 has 2537 Coding DNA Sequences (CDS) for proteins where 319 could be found in the optimized experiment. When using the alternative approach with used vs. unused cell culture medium, 794 proteins were found. This can indicate a higher degree of cellular lysis, but it can also be due to a higher number of cells added to the Flow Cell, where signals from "unwanted" proteins are strengthened, masking the signals from virulence or adhesion proteins.

Of the six virulence and/or adhesion proteins found among the 18 strongly upregulated proteins in the HaCaT colonisation model, all were also found in the used cell culture model, even though the up-and downregulation could be different between the methods; three YSIRK/LPXTG domains (2 strongly upregulated, 1 slightly upregulated), one heme oxygenase (slightly upregulated), AtlE (strongly upregulated) and SceD (slightly downregulated). The results indicate that the HaCaT cells or factors produced from HaCaT cells influence the up- and downregulation of proteins of bacteria in both models, but direct contact might influence the bacteria differently. LysM- or CHAP domain proteins could also be found in the used cell culture model. A nuclease (strongly

upregulated), not previously described for the HaCaT colonisation model was found among the extracellular proteins in the used cell culture medium model. Nuc1 nuclease in *S. aureus* allows the bacterium to avoid neutrophil extracellular trap (NET)-mediated killing (Schilcher et al., 2014).

The expression of CWA proteins can be altered by growth conditions (Foster et al., 2014), and the growth conditions between the methods were different. The comparison of protein results in the alternative approach was done between overnight cultures in used and unused cell culture medium. The bacterial solutions were maybe not as homogenous as for samples run in the HaCaT colonisation model. The OD-values for samples added to the LPI™ Flow Cell were equal, but the OD does not reflect if the bacteria are alive or lysed. The growth of bacteria in overnight cultures in used medium was better than in unused medium, indicating different growth conditions. Factors in the used medium influenced the growth, making the bacteria grow faster. In the HaCaT colonisation model, only bacteria that bind to HaCaT are analysed. That might also explain differences in protein results between the methods.

All strong and slightly upregulated proteins (59 of 319) in the HaCaT colonisation model were compared concerning distribution of up-and downregulation of the same proteins in the alternative approach, and the results were not the same. Only 16 of the compared proteins in the alternative approach were strong or slightly upregulated. The rest was downregulated (16), not found (3) or did not show any up- or downregulation (24).

In the HaCaT colonisation model, more proteins were classified as involved in: Translation, ribosomal structure and biogenesis; Posttranslational modification, protein turnover, chaperones; Energy production and conversion; Carbohydrate transport and metabolism. In the alternative approach, more proteins were grouped as involved in: Amino acid transport and metabolism; Nucleotide transport and metabolism; Coenzyme transport and metabolism; Transcription; Replication, recombination and repair; function unknown. The highest number of proteins in the HaCaT colonisation model was found in the COG groups translation, ribosomal structure and biogenesis, and posttranslational modification, protein turnover, chaperones, maybe indicating an actively growing cell.

5.4 Comparison with other methods

Even though the predicted cytoplasmic protein rate was high in the HaCaT colonisation model (66% of 319 proteins), the rate was within the expected range. The rate of predicted cytoplasmic proteins in bacteria when performing surface shaving is extremely variable (e.g. 0%-70), and is also variable when performing surface shaving of different strains of the same species (Olaya-Abril et al., 2014). As an example, the designers of the second-generation surface shaving approach predicted 6% cytoplasmic proteins of 72 *S. pyogenes* proteins identified in total (Rodriguez-Ortega et al., 2006). In their experiment, exponentially growing bacteria were collected and treated with either trypsin or proteinase K for 30 minutes to shave the bacterial surface of exposed protein domains (25% of the proteins were found in common to both protease digestions) (Rodriguez-Ortega et al., 2006). When Ventura and co-workers performed surface protein analysis of the *S. aureus* USA 300 strain in the late-exponential phase of growth *in vitro*, 113 proteins were identified, and 55% of them were from cytoplasmic origin, the rest were cell wall, extracellular or membrane proteins (Ventura et al., 2010).

Gram-positive bacteria have a cell wall in contrast to Gram-negative bacteria. The cell wall is assumed to be protective to lysis. Actually, the predicted cytoplasmic rate of Gram-negatives might be similar to what is found in Gram-positive analyses (Olaya-Abril et al., 2014). In an experiment with *E. coli*, 10% of the identified 86 proteins after surface shaving were predicted to be cytoplasmic (Cirulli et al., 2007).

As cytoplasmic proteins are often found when performing surface protein shaving of organisms, combining proteomics with bioinformatics can be an important tool when for instance searching for possible vaccine candidates. The *in silico* analysis of our selected strain is currently in progress.

A false-positive control strategy for Gram-positive cell surfaceomics has been suggested by Solis and Cordwell to better control for cellular lysis and the release of intracellular proteins (Solis and Cordwell, 2016, Solis et al., 2010). Their samples and false controls are centrifuged after incubation (with or without protease) to remove whole cells, and LC-MS/MS is performed on the supernatant. Even though their approach is not directly

comparable to our method, a false-positive control strategy could possibly have been included in our protocol, even though the method would be more time consuming.

The cytoplasmic proteins might be due to cellular lysis, moonlighting proteins or MV structures (Olaya-Abril et al., 2014, Solis and Cordwell, 2016). Several species produce MVs (Olaya-Abril et al., 2014), including staphylococci (Askarian et al., 2018). MVs can also be found in *S. haemolyticus* (our lab, unpublished results). A significant reduction of cell viability after protease treatment has never been found (Olaya-Abril et al., 2014). If cells are in the exponential phase of growth and undergo active division, the rate of cell death is low (Olaya-Abril et al., 2014).

In our HaCaT colonisation model, protease treatment was not done directly on cells in exponential phase of growth, but the cells were presumably in exponential phase when sorted on FACS. After bacterial colonisation of HaCaT cells in cell culture medium, cells were sorted on FACS (2.5 hours) and centrifuged (1 hour) prior to immobilisation (35 minutes) and protease treatment (20 minutes) in the LPI™ Flow cell. The duration from handling overnight cultures to protease treatment could possibly influence cell viability. In the alternative approach, samples were added to the LPI™ Flow Cell shortly after overnight incubation (possibly late exponential phase or stationary phase). A higher grade of predicted cytoplasmic proteins could be found using the alternative approach compared to the HaCaT colonisation model, maybe indicating a higher grade of lysis. Cells sorted on FACS would have a quite uniform shape, and destroyed/lysed cells would possibly not be part of the sample for protease treatment.

When sorting bacteria on FACS, the bacterial particles were collected in PBS. Samples were kept in PBS during centrifugation before protease treatment in the LPI™ Flow Cell. Prolonged incubation in PBS and centrifugation could possibly influence the cell viability. However, the viability of prolonged incubation in PBS was tested, and CFU/ml did not decrease over time.

The LPI approach to surface shaving differs from other methods by protein immobilising of whole cells within a flow cell prior to digestion, where no detergent or sample clean up is needed prior to downstream analysis. One of the advantages is that the environment around the proteins can be changed without loss or dilution of the sample.

The LPI approach has previously been used for surface shaving of *Staphylococcus*, *Streptococcus* and *Lactobacillus*, however, results are in preparation (personal communication with Nanoxis Consulting AB), and it is difficult to say whether the protein results retrieved in our experiment are representative using this technique. Elucidation of the outer membrane proteome of *Salmonella enterica* serovar Typhimurium using the LPI technique has been done where they generated outer membrane vesicles for isolation of outer membrane proteins (Chooneea et al., 2010), but this technique is not comparable to ours.

Use of TMT-labelling enabled relative quantification between peptide samples that were uniquely labelled and thereafter pooled for Mass Spectrometry (MS). The use of TMT-tags made it possible to look at up- and downregulation of proteins comparing two conditions. The protein expression of bacteria when colonising HaCaT cells was compared to the expression when treating bacteria in the same manner except for the HaCaT colonisation step.

A combination of methods for protein extraction was not done in our experiment, which might have retrieved more interesting protein results. Romero-Saavedra and co-workers have described a combination of three different extraction methods for surface exposed proteins of *Enterococcus faecium*: trypsin shaving, biotinylation and elution at high pH (Romero-Saavedra et al., 2014). The total proteins found and predicted extracytoplasmic localization (parenthesis) with each method was 390 (10%) by trypsin shaving, 329 (15%) by elution at high pH and 45 (63%) by using biotinylation. Six proteins were predicted to be surface exposed that were detected with all three methods (Romero-Saavedra et al., 2014). A combination of methods has also been described for the cell surface proteome profiling of *S. aureus* using a combination of $^{14}N^{15}N$ metabolic labelling, biotinylation and LC-MS/MS approaches (Hempel et al., 2011, Hempel et al., 2010). Various extraction times with the protease or the use of several different kinds of proteases could also have given us more interesting results.

5.5 Limitations of the method

Even though the method for surface protein shaving of bacteria has advantages (e.g. bacterial contact with human cells), the method also has limitations. One of them is that

the method is time consuming; it took more than 10 hours of work for one bacterial sample in the main experiment, starting by measuring OD of overnight cultures to the sample was finished in the LPI™ Flow Cell. In addition several working hours was done during days and weeks beforehand with preparations of human cells and bacterial cultures. This leads to a low throughput of samples, especially if biological replicates are made. Only one strain of *S. haemolyticus* was chosen for the main experiment to make the method feasible, and to be able to finish the main laboratory experiments with the LPI™ Flow Cell during some days of work. If the method had a high throughput of samples, protein results from different bacterial strains could have been compared, or even more samples from the same strain to be sure that the results were reproducible. In addition, a false positive control could have been included to account for cytoplasmic proteins from lysed cells.

Some of the main problems with the method are the high inoculum of bacteria needed for the LPI™ Flow Cell and the time consuming sorting of cells by FACS. A high concentration of bacterial cells was necessary to get results from the protein analysis. The concentration of samples (CFU/ml) collected from FACS did not vary much, because one droplet of PBS containing one particle / bacterial cell was sorted at the time, resulting in large volumes after FACS in order to get enough sample material for the LPI™ Flow Cell. The duration of separation of one sample on FACS was about 2.5 hours in addition to preparations beforehand followed by ultracentrifugations afterwards before the sample could be transferred to the LPI™ Flow Cell. The implementation of two centrifugation steps (>1 hour duration) in ultracentrifuges after FACS to concentrate the sample, made the method even more time consuming. More than one sample could be run on the LPI™ Flow Cell on one day, but only one sample at a time on FACS, limiting sample throughput.

Even though the reproducibility was good in the growth experiments, the exact concentration of bacterial cells added to HaCaT cells was not known before the next day after serial dilutions and CFU count.

In the last surface protein experiment (samples 1-6), the concentration of bacteria (CFU/ml) added to the LPI™ Flow Cell was not determined because all of the material after ultracentrifugation was added to the flow cell (except for one sample). Further

optimization of the flow cells where less sample material is needed is currently in preparation by Nanoxis Consulting AB.

Even though precautions were taken to avoid unnecessary chemical exposure of cells, e.g. mechanical instead of chemical detachment of eukaryotic and bacterial cells from tissue culture plates, a few chemicals still had to be used to run the experiment. Vancomycin BODIPY™ was for instance used to be able to sort *S. haemolyticus* from HaCaT cells on FACS. The conjugate should technically stain all Gram-positive bacteria that are sensitive to vancomycin. Even though previous proteome analyses of labelled *S. aureus* have revealed that the labelling procedure with Vancomycin BODIPY™ provoked only minor changes at the proteome level (Hildebrandt et al., 2016), the conjugate still binds to and changes the bacterial cell wall. There could also be differences when using the conjugate in different species (e.g. *S. haemolyticus* vs. *S. aureus*). Labelling with Vancomycin BODIPY™ was not necessary when running LPI™ Flow Cell with secondary cultures of *S. haemolyticus* in used cell culture medium. By using this method, the bacteria were not in direct contact with eukaryotic cells, but the number of chemical and mechanical exposures were reduced, and could be one of the reasons for the differences we saw in protein expression between the two experiments.

Although trying to imitate the conditions for bacterial and eukaryotic cells in normal settings in the environment when performing the methods, the experiments are still done *ex vivo*, lacking *in vivo* results.

Keeping bacterial lysis as low as possible is important when performing surface shaving (Solis and Cordwell, 2016). As previously described, proteins related to the cytoplasm could be found when performing the HaCaT colonisation surface shaving experiment. This could be related to lysis of cells, but could also originate from moonlighting proteins or proteins released by shedding membrane-vesicles structures (Solis and Cordwell, 2016, Olaya-Abril et al., 2014).

In addition to the HaCaT colonisation model, an alternative approach with used cell culture medium was performed. If the protein results were the same, the method with used medium would have been much easier to perform, with a higher sample throughput. Even though some similar protein results were found, the results were not

directly comparable. More proteins were found in the alternative approach, maybe indicating a higher grade of cellular lysis.

Another limitation, not of the method as such, but of the protein analysis is the expenses when using TMT tags. A high number of samples lead to high costs.

5.6 Future aspects

Developing and running the surface protein shaving experiment was time consuming, but provided us with new knowledge about proteins on the surface of our *S. haemolyticus* strain. The next step will be to analyse the data in more detail. Functional assays can be performed to find the proteins mode of action. It would also be interesting to analyse both clinical and commensal strains of *S. haemolyticus*, to look at the differences in protein expression. The developed method with cell sorting on FACS before running proteomics might also be used to find expressed *S. haemolyticus* proteins in blood.

6 Conclusion

A method to identify *S. haemolyticus* surface proteins expressed during colonisation of human keratinocytes was developed. This is to our knowledge the first described experiment for surface shaving of expressed staphylococcal proteins after direct contact with eukaryotic cells. Our hypothesis was that *S. haemolyticus* would express specific surface proteins important for adhesion to the host upon contact with human cells. Proteins were differentially expressed, and several adhesion and virulence proteins were upregulated upon *S. haemolyticus* colonisation of HaCaT cells. Among the 18 strongly upregulated proteins of the 319 found in total, six adhesion and/or virulence proteins were found: three proteins with YSIRK/LPXTG motifs, one reported important for attachment to nares of rats, one AtlE protein and one heme oxygenase. The combination of HaCaT colonisation and surface shaving methods is an important tool for examining host-microbe interactions. 66% of the proteins were predicted to be from cytoplasmic origin. Even though the rate is high, it is within the expected range. Another approach or a combination of strategies for surface shaving might have detected more interesting proteins. Still, the method has provided us with new knowledge about proteins on the surface of our *S. haemolyticus* strain, and functional assays can now be performed to find the proteins mode of action. Some of the results from the alternative used cell culture medium approach were similar to the HaCaT colonisation approach, but the methods were not directly comparable. More proteins in the alternative approach could indicate a higher degree of cellular lysis.

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Appendix

Surface shaving of expressed bacterial proteins, optimized experiment

Table 17 - Transferring HaCaT cells in 6 well tissue culture plates after growing in four T75 cell culture flasks.

Counted cells x 10 / ml	**Mean value**	**Total volume to tubes with cell suspension to get 10^5 / ml cells:** $\frac{Counted\ cells\ x\ Volume\ of\ cell\ suspension}{Concentration\ of\ cells\ that\ we\ want}$
1.18 x 10^6	1.08 x 10^6	$\frac{10.8x10^5 \times 37\ ml}{2\ x10^5} = 200\ ml$
1.09 x 10^6		
1.00 x 10^6		
1.06 x 10^6		

Table 18 - Defining CFU/ml of different samples (samples 1-12) in the optimized surface shaving experiment. For sample 2,3 and 6, the volume was too large after the first round of ultracentrifugation with small ultracentriguge tubes and rotor SW 50.1. Samples were centrifuged again with the same conditions to increase the concentration of bacteria added to the LPI™ Flow Cell.

Type of sample **53-38**	**Sample no.**	**Dilution**	**Number of colonies on blood agar plates after dilution**							**CFU/ml**	**CFU/ml, mean value**
			0	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**		
Scraped HaCaT and *S. haemolyticus* from 6 well cell culture plates	1	A	Dense	Dense	Dense	Dense	57	6	0	2.3 x 10^7	1.8 x 10^7
		B	Dense	Dense	Dense	Dense	35	4	0	1.4 x 10^7	
Supernatant after FACS and ultra centrifugation	1	A	0	0	0	0	0	0	0	0	20
		B	1	0	0	0	0	0	0	40	
Wash liquid from flow cytometry by Nanoxis	1	A	Ca. 200	0	0	0	0	0	0	8 000	8 000
Overnight culture in DMEM with 10% FBS	2	A	Dense	Dense	Dense	Dense	27	1	0	1.1 x 10^7	1.2 x 10^7
		B	Dense	Dense	Dense	Dense	32	3	0	1.3 x 10^7	

Concentration of *S. haemolyticus* added to HaCaT cells	2	A	Dense	Dense	Dense	Dense	35	3	1	1.4×10^7	1.7×10^7
		B	Dense	Dense	Dense	Dense	48	2	0	1.9×10^7	
Scraped HaCaT and *S. haemolyticus* from 6 well cell culture plates	2	A	Dense	Dense	Dense	Dense	65	7	3	2.6×10^7	2.7×10^7
		B	Dense	Dense	Dense	Dense	70	11	0	2.8×10^7	
Supernatant after FACS and ultra centrifugation	2	A	8	0	0	0	0	0	0	320	460
		B	15	1	1	0	0	0	0	600	
Wash liquid from flow cytometry by Nanoxis	2	A	Dense	75	12	0	0	0	0	30 000	30 000
		B	Dense	75	7	0	0	0	0	30 000	
Overnight culture in DMEM with 10% FBS	3	A	Dense	Dense	Dense	Dense	44	4	1	1.8×10^7	1.8×10^7
		B	Dense	Dense	Dense	Dense	45	6	0	1.8×10^7	
Scraped HaCaT and *S. haemolyticus* from 6 well cell culture plates	3	A	Dense	Dense	Dense	Dense	29	3	1	1.2×10^7	1.2×10^7
		B	Dense	Dense	Dense	Dense	31	3	0	1.2×10^7	
Wash liquid from flow cytometry by Nanoxis	3	A	Dense	24	1	0	0	0	0	9 600	11 600
		B	Dense	34	3	0	0	0	0	13 600	
Overnight culture in DMEM with 10% FBS	4	A	Dense	Dense	Dense	Dense	49	6	1	2.0×10^7	2.1×10^7
		B	Dense	Dense	Dense	Dense	55	10	1	2.2×10^7	
FACS liquid	4	A	Dense	Dense	52	2	2	0	0	2.1×10^5	2.0×10^5
		B	Dense	Dense	49	7	0	0	0	2.0×10^5	
Wash liquid from flow cytometry by Nanoxis	4	A	93	8	1	0	0	0	0	3 720	3 060
		B	60	3	0	0	0	0	0	2 400	
Overnight culture in	5	A	Dense	Dense	Dense	Dense	42	13	0	1.7×10^7	1.8×10^7

DMEM with 10% FBS		B	Dense	Dense	Dense	Dense	50	3	0	2.0×10^7	
Supernatant after FACS and ultra centrifugation	5	A	2	0	0	0	0	0	0	80	60
		B	1	1	0	0	0	0	0	40	
Wash liquid from flow cytometry by Nanoxis	5	A	28	9	0	0	0	0	0	1 120	1 480
		B	46	8	0	0	0	0	0	1 840	
Overnight culture in DMEM with 10% FBS	6	A	Dense	Dense	Dense	Dense	53	8	1	2.1×10^7	2.1×10^7
		B	Dense	Dense	Dense	Dense	52	8	1	2.1×10^7	
FACS liquid	6	A	Dense	Dense	50	1	1	1	0	2.0×10^5	2.0×10^5
Supernatant after FACS and ultra centrifugation	6	A	0	0	0	0	0	0	0	0	0
		B	0	0	0	0	0	0	0	0	
Resuspended pellet after ultra centrifugation*	6	A	-	Dense	Dense	97 / (194)	10	1	0	7.8×10^6	7.8×10^6
Wash liquid from flow cytometry by Nanoxis	6	A	51	4	2	0	0	0	0	2 040	2 120
		B	55	3	1	0	0	0	0	2 200	
Overnight culture in <u>used</u> DMEM with 10%FBS	7	A	Dense	Dense	Dense	Dense	Dense	98	8	3.9×10^8	3.5×10^8
		B	Dense	Dense	Dense	Dense	Dense	76	8	3.0×10^8	
Overnight culture in <u>used</u> DMEM with 10%FBS	8	A	Dense	Dense	Dense	Dense	Dense	117	15	4.7×10^8	4.5×10^8
		B	Dense	Dense	Dense	Dense	Dense	107	16	4.3×10^8	
Overnight culture in <u>used</u> DMEM with 10%FBS	9	A	Dense	Dense	Dense	Dense	Dense	97	11	3.9×10^8	3.7×10^8
		B	Dense	Dense	Dense	Dense	Dense	90	9	3.6×10^8	
Overnight culture in	10	A	Dense	Dense	Dense	Dense	32	3	2	1.3×10^7	1.5×10^7

DMEM with 10% FBS		B	Dense	Dense	Dense	Dense	44	4	0	1.8×10^7	
Overnight culture in DMEM with 10% FBS	11	A	Dense	Dense	Dense	Dense	57	3	0	2.3×10^7	2.2×10^7
		B	Dense	Dense	Dense	Dense	50	12	1	2.0×10^7	
Overnight culture in DMEM with 10% FBS	12	A	Dense	Dense	Dense	Dense	59	11	0	2.4×10^7	2.6×10^7
		B	Dense	Dense	Dense	Dense	70	8	0	2.8×10^7	

*Only 10 µl was available for the first dilution in 180 µl of this sample from stock to 10^{-1} dilution. After that the dilution was done by adding 20 µl to 180 µl. 97 colonies were counted at 10^{-3} dilution, and the expected result would have been about 194 colonies if 20 µl of sample had been available for the first dilution.