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Differential involvement of glycans in the binding of *Staphylococcus epidermidis* and *Corynebacterium* spp. to human sweat

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Abstract

Sweat is a secretory fluid that can be a source of unpleasant body odour due to interaction of resident bacteria with sweat components. Identification of glycoproteins in sweat suggests that protein-conjugated glycans may act as binding epitopes for bacteria, as found in other secretory fluids such as human milk, tears and saliva which help to protect epithelial surfaces from infection.

We conducted proteomic and glycomic analysis of sweat to reveal an abundance of glycoproteins, predominantly carrying bi-antennary sialylated *N*-glycans with or without fucose. A fluorescent plate assay was used to determine whether glycans on sweat proteins provide binding epitopes for odour-producing skin commensals *Staphylococcus epidermidis* and *Corynebacterium*. Sialic acid and fucose were found to be important binding epitopes for *S. epidermidis* 3-22-BD-6, a strain recently isolated from human sweat, whereas fucose (but not sialic acid) contributed to the binding of Type strain *S. epidermidis* ATCC 12228. In contrast, our results indicate that sweat *N*-glycans do not provide binding epitopes for *Corynebacterium*.

Synthetic sugar mimics of Lewis blood group antigens were investigated as potential inhibitors of the binding of *S. epidermidis* 3-22-BD-6 to sweat. Pre-incubation of the bacterium with LeB, LeX, LeY and sLeX (pentaose) resulted in a significant reduction in sweat protein adhesion indicating that terminal fucose is a key binding epitope, particularly when linked to a Type 2 chain (Gal β 1-4GlcNAc) configuration (LeY).

Our results form an impetus for future studies seeking to elucidate the role of glycans in sweat associated malodour, with possible implications for cosmetic and medical fields.

Keywords: sweat; glycan; glycoprotein; fucose; *Staphylococcus*; *Corynebacterium*

Introduction

The human body is home to an abundance of microorganisms, most of which live with us in a commensal relationship. However, some microorganisms can cause disease by attaching to epithelial surfaces, then invading cells and tissues and/or releasing toxins and interfering with cellular functions (Kato and Ishiwa, 2015; Sakarya and Öncü, 2003; Szymanski *et al.*, 2017). Research suggests that secretory fluids may help to prevent the primary attachment phase of pathogens at least partially via sugars attached to secretory glycoproteins. The sugars (glycans) on the secreted glycoproteins in human milk, saliva and tears are thought to mimic the glycan moieties on the epithelial surfaces of the intestinal tract, oral cavity and eye, respectively (Everest-Dass *et al.*, 2012; Kautto *et al.*, 2016; Peterson *et al.*, 2013). Acting as molecular decoys, the secreted glycoproteins potentially attach to the would-be pathogens and flush them away before attachment to the host cell surfaces and subsequent infection occur.

Microorganisms differ in their adhesion mechanisms and often show preference for particular glycan moieties of the host according to the specific affinities of the microbial cell surface glycan-binding proteins or lectins. For instance, the lectins PA-IL and PA-IIL of *Pseudomonas aeruginosa* bind to the sugars galactose and fucose, respectively (reviewed in Venkatakrishnan *et al.*, 2013), while the gastrointestinal pathogens *Helicobacter pylori* and Norwalk virus bind to sugar configurations characteristic of Lewis and H blood group antigens (Hage *et al.*, 2015; Huang *et al.*, 2003; Magalhaes and Reis, 2010; Nasir *et al.*, 2017). Adding to the complexity, variations have also been found in glycan binding specificity between different strains of the same species (Chionh *et al.*, 2009; Kalograiaki *et al.*, 2016; Kautto *et al.*, 2016).

Human skin permanently harbours a vast array of bacteria including *Staphylococcus*, *Corynebacterium* and *Propionibacterium* species (Taylor *et al.*, 2003). Generally, these symbiotic / commensal bacteria are nonpathogenic but may cause infections when skin is compromised (Bhatia *et al.*, 2004; Bowler *et al.*, 2001; Bratcher, 2012). *Staphylococcus epidermidis*, *Corynebacterium* and *Propionibacterium* can also form biofilms on biomedical materials (e.g., tubing and prosthetics), a phenomenon that is becoming one of the leading causes of hospital acquired infections (Tauch *et al.*, 2005; Otto, 2014).

Sweat, the secretory fluid that regularly bathes human skin, provides some protection for the skin via at least components such as antimicrobial peptides and salts (Park *et al.*, 2011). Our recent review (Peterson *et al.*, 2016) has revealed that sweat also contains secreted glycoproteins that seem likely to play a role in providing molecular decoys for microbial adhesion as discovered in other

secretory fluids, but the interaction between bacteria and the glycan moieties in sweat has not been well described.

The moist underarm region, or axilla, provides a niche to *Staphylococcus* and *Corynebacterium* species, also known to produce undesirable odours via the metabolism of human sweat (Bawdon *et al.*, 2015; Hara *et al.*, 2014; Leyden *et al.*, 1981; Taylor *et al.*, 2003; Troccaz *et al.*, 2004). *Staphylococcus* and *Corynebacterium* species are involved in the formation of the pungent sulfur compounds from sweat including hydroxyalkanethiols released from L-cysteine conjugates via the action of carbon-sulfur (C-S) lyases (Natsch *et al.*, 2004; Troccaz *et al.*, 2004). The generation of the odoriferous isovaleric acid in sweat has been largely attributed to the transformation of leucine and branched chain amino acids by *Staphylococcus* species (James *et al.*, 2013; Leyden *et al.*, 1981). However, it is the population density of *Corynebacterium* that has been most closely correlated with magnitude of foul body odour, according to recent comprehensive reviews by Natsch (2017) and Callewaert *et al.* (2017).

Here, the involvement of glycans in the adhesion of the skin-associated bacteria *S. epidermidis*, *C. mucifaciens*, and *C. jeikeium* to human sweat was investigated, and sweat-isolates and laboratory type strains were compared. Moreover, this work investigates a possible involvement of glycans in the attachment mechanisms of skin bacteria to sweat, analogous to that seen in other secretory fluids by which the epithelial surfaces of the human body are protected from infection. Glycan involvement in the interaction between skin bacteria and sweat proteins may have implications for future research and development in the cosmetic industry, and could lead to alternative preventative or treatment options in health and medical fields.

Material and Methods

Sweat collection and preparation

Sweat samples were collected from armpit volunteers by DermScan Laboratoire on behalf of L'Oréal, France, Study number 12E2707 - ACR/PACKO/1250. The procedures for the study were carried out in compliance with the Huriet Law (Law 2004-806 of 09/08/2004) relating to public health and Good Clinical Practices (ICH Topic E6 ref: CPMP/ICH/135/95) defined in the *Bulletin Officiel* (French government gazette) published by the Ministry of Social Affairs and Employment. The research also meets the requirements set out in the Australian National Statement on Ethical Conduct in Human Research (2007) (the current National Statement; Reference No: 5201400088, 27 February 2014).

Sweat samples (~2.5 mL) were desalted using a PD-10 column (GE Healthcare) and dried in a SpeedVac centrifugal vacuum concentrator (Thermo Fisher Scientific). Samples were reconstituted in 500 μ L water and protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Individual concentrated and desalted samples were subjected to proteomic and glycomic analyses. Samples were pooled for the bacterial binding assay.

Proteomic profile of sweat samples

Proteins (10 μ g) was dissolved in 50 mM ammonium bicarbonate and reduction was conducted by adding dithiothreitol (DTT) to final concentration of 5 mM at 37 °C for 1 hour. Alkylation was conducted by adding iodoacetamide (IAA) to final concentration of 10 mM at room temperature for 1 hour. 0.5 μ g sequencing grade trypsin was added and incubated at 37 °C for 16 hours. The peptide mixtures were desalted by hand-made desalting tips. The C18 tips were prepared by punching out small disks of an Empore C18 filter (pore size 60 Å, Empore, 3M) and fitting into 200 μ L pipet tips. C18 tips were washed three times with 20 μ L of 100% acetonitrile (ACN), three times with 20 μ L of 50% (v/v) ACN in 0.1% formic acid, and equilibrated with 50 μ L of 0.1% (v/v) formic acid. After sample loading, tips were washed three times with 20 μ L of 0.1% formic acid. Peptides were eluted by 20 μ L of 60% (v/v) ACN in 0.1% formic acid and 20 μ L of 90% (v/v) ACN in 0.1% (v/v) formic acid and dried. Samples were reconstituted in 2% (v/v) ACN in 0.1% (v/v) formic acid. Peptide mixture equal to 5 μ g was injected for analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) on an Sciex triple TOF 5600 instrument. Peak lists were generated by ProteinPilot (Sciex) and Scaffold (version Scaffold_4.2.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if there were at least two matched peptides above 95% confidence and they could be established at greater than 99.0% probability assigned by the Protein Prophet algorithm using Scaffold.

N-glycan release and MS analysis

Sweat proteins (10 μ g) were reduced by a final concentration of 5 mM dithiothreitol (DTT) at 37 °C for 1 hour, and then alkylated by a final concentration of 10 mM iodoacetamide (IAA) in the dark at room temperature for 1 hour. The protein samples were then spotted onto a polyvinylidene fluoride (PVDF) membrane and *N*-glycans were released according to our standardised protocol (Jensen *et al.*, 2012). Briefly, membranes were washed by water and blocked by 1% polyvinylpyrrolidone (PVP40, Sigma). 2.5 U of PNGase F (Roche) was added to each sample and the reaction was conducted at 37 °C overnight. *N*-glycans were harvested from solution and were reduced by 1 M sodium borohydride in 50 mM potassium hydroxide at 50 °C for 3 hours. The

samples were desalted by Dowex X8 cation exchange resin and clean-up was carried out using a hand-packed porous graphitised carbon tip. Glycan profiles were analysed by LC-MS/MS.

Bacterial strains

Two *Staphylococcus epidermidis* strains were used: *S. epidermidis* 3-22-BD-6 is an odour-producing clinical strain isolated from human sweat by L'Oréal Laboratories, France; *S. epidermidis* ATCC 12228 is a non-biofilm-forming laboratory Type strain that has been used for decades in the food production industry for detection of residual antibiotics (Zhang *et al.*, 2003). Two different species of *Corynebacterium* were also studied: *C. mucifaciens* 3-8BG-6 is an odour-producing clinical strain isolated by L'Oréal Laboratories from human sweat; *C. jeikeium* CIP 103337T (ATCC 43734T) is a laboratory Type strain originally isolated from blood (Jackman *et al.*, 1987).

For clinical sample collection strains, 23 healthy Caucasian men (21-50 years old) were recruited to provide samples. For sample collection, sterile cotton swabs were soaked in 5.0 ml of sodium chloride (NaCl) 0.15M – Tween 20 0.1% buffer. A 16 cm² area was sampled by rubbing twice the surface for 60 sec. The head of each swab was cut from the handle, placed into the tube containing the buffer. According to the national "Arrêté du 11 mai 2009 relatif aux définitions de certaines catégories de recherches biomédicales", this non-interventional study without tested product nor invasive assessment method did not require Regulatory Approval. However, the study was conducted in compliance with the World Medical Association Declaration of Helsinki, national and EU regulations and L'Oréal Research and Innovation's procedures based on ICH guidelines for Good Clinical Practice. All volunteers received verbal and written information concerning the study in accordance with the applicable local regulations, guidelines and the current SOP. This information explained the nature, purpose and risks of the study and emphasised that participation in the study was voluntary and that the volunteer might withdraw from the study at any time and for any reason. The volunteer's written informed consent to participate in the study was obtained prior to any study related procedure being carried out. According to national law "Informatique et liberté" dated January 6th 1978, modified by law No 94-548 dated July 1st 1994, and law n° 2004-801 dated August 6th 2004, the subject database is declared to the "Commission Nationale de l'Informatique et des Libertés (the national commission of data processing and freedom).

Bacterial binding assay

Bacteria in glycerol stock were plated on Brain Heart Infusion (BHI) agar plates and cultured in semi-anaerobic conditions at 37°C overnight. Colonies were inoculated into 5 mL BHI broth and cultured at 37°C overnight prior to use in the binding assay.

The binding assay was modified from a 96-well plate assay previously established in our laboratory in which a protein of choice is immobilised into the wells of a polyvinylidene fluoride (PVDF) filter plate, then probed by fluorescently labelled bacteria (Kautto *et al.*, 2016). Our usual protocol involves the application of 50 μ L of 1 mg/ml protein to the PVDF wells of the 96 well assay plate to enable full coverage of the PVDF membrane with our protein of choice, prior to assessment of bacterial adhesion. We have found that many bacterial species strongly bind to PVDF, so a complete blocking of the membrane is important. However, the low concentration of protein in sweat samples (0.1 – 0.5 μ g/ml) and the limited amount of sweat available prevented us from using 1 mg/ml sweat protein concentration to fully cover the PVDF wells. To avoid binding of bacteria to non-occupied PVDF membrane, several standard proteins were tested as blocking agent. The *S. epidermidis* and *Corynebacterium* strains showed the lowest binding affinity toward bovine whey protein, which was consequently used as a blocking agent in the assays reported here.

To immobilise sweat proteins in the 96-well PVDF plate, 10 μ g sweat protein in 50 μ L Phosphate Buffered Saline (PBS) was added into each well and incubated for 30 minutes. After washing 3 times with PBS, 50 μ L whey protein solution (1 mg/mL) was added into each well and incubated for 30 minutes to block non-occupied PVDF membrane. The whey protein solution was then removed and wells washed three times with PBS.

Bacteria were harvested from the liquid cultures and stained by SYBR® Green fluorescent dye (Invitrogen) as described in Kautto *et al.* (2016). Stained bacteria were then added to wells of 96-well plate containing the immobilised sweat proteins (and whey blocker) and incubated for 20 minutes. Each experiment was carried out using triplicates of each sample, and triplicate Controls were also included in which bacteria were applied to wells of 96-well plate containing whey protein only (no immobilised sweat proteins). After washing 3 times with PBS, fluorescence intensity was measured in a microplate reader (FluoStar, BMG Labtech) to enable quantification and comparison of bacterial binding to the immobilised sweat. Student's t test was used for statistical analysis in all bacterial binding assays.

Exoglycosidase digestion

Sweat proteins were prepared at a concentration of 1 μ g/ μ L in 50 mM sodium acetate buffer pH 5.5. 1 μ L of broad specificity sialidase (Prozyme, Sialidase A, 5 mU) or a combination of broad specificity sialidase with α 1-3/4 fucosidase (Calbiochem, 0.1 mU) and α 1-2 fucosidase (Calbiochem, 0.1 mU) were added to 200 μ g sweat protein for digestion overnight at 37 °C overnight. For non-treated control, the same condition was used without adding enzyme(s). Removal of terminal residues was confirmed by LC-MS/MS as described above (*N-glycan release*

and MS analysis). The bacterial binding assay was then used to determine bacterial binding to the exoglycosidase-digested sweat proteins in comparison to non-digested sweat proteins.

De-N-glycosylation

To remove *N*-glycans, 100 µg sweat proteins were first denatured by incubation at 95 °C for 5 minutes in the presence of 0.5% SDS and 40 mM DTT. After chilling, the solution was adjusted to final concentration of 50 mM ammonium bicarbonate pH 8.0 containing 1% Triton X-100. 2 µL PNGase F (2 U) was added and incubated at 37 °C overnight. For a “denatured” control, sweat proteins were denatured as above but no PNGase F was added. For a “native” control, sweat proteins were incubated at 95 °C in 50 mM ammonium bicarbonate pH 8.0 but no PNGase F was added.

Inhibition assay

Wells of a 96-well plate were coated with 10 µg sweat protein and whey was used to block any PVDF membrane that remained exposed, as described above. Fluorescently stained bacteria were pre-incubated with selected glycan-containing compounds (1 mg/mL in PBS) for 15 minutes before adding into wells of 96-well plate. After PBS wash of wells to remove unbound bacteria, fluorescence intensities were measured as described above. Two controls were included in each inhibition assay experiment: 1) bacterial adhesion to wells containing whey blocker only (no sweat proteins); and 2) bacterial adhesion to wells containing sweat proteins following incubation of the bacteria in PBS only (no inhibitor). Fluorescence intensity was measured using the FluoStar microplate reader, as described above (*Bacterial binding assay*).

Results and Discussion

Proteomic and glycomic profiles of sweat

The protein concentrations of individual sweat samples before desalting ranged between 0.1 mg/ml to 0.5 mg/ml. This is higher than reported in the literature (0.06 – 0.12 mg/ml; Lloyd, 2008) but very much lower than the protein concentration in other secretory fluids such as tears (6 – 10 mg/ml; Fullard and Snyder 1990; Ng and Cho, 2000), saliva (1.4 – 6.4 mg/ml; Lloyd, 2008), and human milk (8.9 mg/ml; Rih, 1985). The extremely low concentration of protein in sweat and the limited supply of samples from the clinical study were challenging factors that influenced downstream experimental processes, as described in more detail below.

Sweat proteins from individual samples were subjected to proteomic analysis to gain an overview of the protein constituents and compare to previous research. The proteomic analysis of sweat

confirmed the presence of many glycoproteins, consistent with the results of previous analyses (Park *et al.*, 2011; Peterson *et al.*, 2016; Raiszadeh *et al.*, 2012). The most abundant glycoproteins were Prolactin-inducible protein, Apolipoprotein D, Clusterin, Zinc alpha-2-glycoprotein, Immunoglobulin A1, and Cathepsin (Supplementary Table 1 provides the proteomic profiles of four typical sweat samples).

Glycomic profiles of sweat proteins were conducted to determine possible glycan epitopes for adhesion. MS analysis of the *N*-glycans released from the sweat samples revealed high mannose, hybrid and complex type *N*-glycans. Most of the complex *N*-glycans are bi-antennary structures with sialic acid with or without fucose. Sialylated biantennary structures (m/z at 1111.5 and fucosylated at 1184.5) were typically the most abundant glycan species across all samples analysed (Supplementary Table 2). Overall, the *N*-glycan profile of the sweat proteins was 70.3% sialylated and 18.5% fucosylated. This can be compared to the *N*-glycan profile of glycoproteins in other secretory fluids: human milk, 84% fucosylated, 47% sialylated (Dallas *et al.*, 2011); saliva, more than 84% fucosylated, 21.6% sialylated (Everest-Dass *et al.*, 2012); and tears, 65% fucosylated, 33% sialylated (Nguyen-Khuong *et al.*, 2015).

We note here that the human sweat samples conceivably could have contained glycans derived from the sweat microbiota. However, our proteomic analysis of the sweat samples did not find any microbial proteins, and our glycomic analysis revealed *N*-linked glycan structures typical of human glycoproteins. From this we surmise that there were either no protein-bound glycans derived from microbiota in the sweat samples or, if present, they were below detection levels and thus not likely to be a significant influence on our results.

Binding of *Staphylococcus epidermidis* and *Corynebacterium* species to sweat

We tested if the *S. epidermidis* and *Corynebacterium* strains showed significant binding to human sweat proteins using a modified plate assay, which was originally developed in our laboratory to investigate the binding of bacteria to other secreted glycoproteins such as those in tears and milk (Kautto *et al.*, 2016). Due to the low concentration of protein in sweat and the limited number of samples, we reduced the amount of sweat proteins applied to the PVDF wells and then blocked the membrane with whey protein as described in *Material and Methods*. The whey protein binds to the PVDF via hydrophobic interaction, thus coating the membrane where not enough sweat proteins are present and preventing bacterial binding to the PVDF. Our preliminary work indicated that there was very low bacterial binding to the whey protein in comparison to other potential protein blockers tested, and a control of whey protein without sweat was run in parallel to further validate results. All *S. epidermidis* and *Corynebacterium* strains showed statistically higher binding toward

sweat samples ($p<0.05$) than to the wells containing whey blocker only (“No Sweat Control”, Fig. 1), indicating that these commensal skin bacteria bind preferentially to human sweat proteins.

Figure 1: *S. epidermidis* and *Corynebacterium* species bound to sweat proteins significantly more than to the blocker protein alone (No Sweat Control). Bar chart shows results of the plate assay in which fluorescence intensity is representative of bacterial binding (* $p<0.05$).

Determining glycan epitopes involved in the binding of bacteria to sweat

We investigated the role of glycans in the binding of the *S. epidermidis* and *Corynebacterium* strains to sweat proteins. Glycosidases (sialidase and fucosidase) were used to modify terminal glycan epitopes. Trimming of glycan structures was confirmed by LC-MS/MS analysis (Fig. 2).

Figure 2: MS analysis confirmed action of glycosidases used to modify terminal glycan epitopes on the sweat glycoproteins. Here, the peaks of sialylated glycans were trimmed to the corresponding non sialylated complex type N-glycans. Action of fucosidases to remove terminal fucose was confirmed similarly.

Following removal of sialic acid, the clinical *S. epidermidis* 3-22-BD-6 strain showed decreased binding to the sweat proteins ($p = 0.01$). However, the adhesion of this strain to the desialylated sweat glycoproteins was still greater than the Control (No Sweat Proteins) indicating another mechanism of adhesion to sweat in addition to via sialic acid (Fig. 3). In contrast, there was no difference between the binding of *S. epidermidis* ATCC 12228 toward sialidase treated and non-treated sweat proteins indicating that sialic acid is not critical for the adhesion of the laboratory type strain to sweat (Fig. 3). No significant change was detected in the binding of either of the *Corynebacterium* strains to sweat following removal of sialic acid.

Figure 3: Removal of terminal sialic acid from sweat glycoproteins significantly reduced binding of *S. epidermidis* 3-22-BD-6 to sweat proteins ($*p < 0.05$), whereas the binding of ATCC 12228 was not significantly reduced. No Sweat Proteins = binding to blocker only.

Sweat samples were also treated with a combination of broad specificity sialidase and fucosidases (both $\alpha 1$ -2 specific and $\alpha 1$ -3/4 specific). The reason to combine sialidase with fucosidase is because terminal sialic acid can act as structural hindrance to block the action of fucosidase. Binding assays were conducted to test the effect of the removal of these terminal glycans from sweat on the binding of bacteria.

Figure 4: Removal of terminal sialic acid and fucose from sweat glycoproteins significantly reduced binding of *S. epidermidis* ATCC 12228 (* $p < 0.05$), but there was no statistical difference in the binding of *S. epidermidis* 3-22-BD-6. Fluorescence intensity is representative of bacterial binding. No Sweat Proteins = binding to blocker only; Sweat (-) = binding to sweat proteins; Sweat (+) = binding to sweat proteins treated with sialidase and fucosidase.

After trimming of fucose and sialic acid from the sweat *N*-glycans, the binding of *S. epidermidis* ATCC 12228 decreased to a level that was not significantly different from the blocker only (“No Sweat Proteins”, Fig. 4), suggesting that binding to the sweat proteins had been essentially abolished. Since removal of sialic acid alone had no statistical effect on the binding of *S. epidermidis* ATCC 12228 to sweat (Fig. 3), the results in Figure 4 suggest fucose constitutes the major factor enabling the ATCC strain to bind to sweat samples.

There was not a statistically significant reduction in the binding of *S. epidermidis* 3-22-BD-6 to the sweat proteins following sialidase and fucosidase treatment (Fig. 4). This seemed contrary to the significant reduction in binding of 3-22-BD-6 to sweat proteins following removal of sialic acid alone (Fig. 3). We speculated that removal of both sialic acid and fucose could perhaps have exposed other, as yet untapped, binding interactions with the sweat proteins particular to the 3-22-BD-6 strain, which could have compensated for the loss of binding to sialic acid and (potentially) fucose. Though, on close analysis, our experimental data revealed a substantial reduction in the binding of two of the three technical replicates (a reduction consistent with and/or additive to that shown in Fig. 3 when only sialic acid was removed), giving us reason to suspect a possible outlier or technical error in our results from the third replicate. Repetition of this experiment was not possible due to the limited number of sweat samples available and the low protein concentration per

sample. Further study of the involvement of both fucose and sialic acid to the binding of *S. epidermidis* 3-22-BD-6 to sweat glycoproteins was undertaken via investigation of these sugars as potential inhibitors of bacterial binding to sweat, as described further below (“Screening of potential inhibitors of *S. epidermidis* adhesion to sweat”).

The binding of *C. mucifaciens* 3-8BG-6 and *C. jeikeium* CIP 103337T was not significantly altered by the removal of sialic acid and fucose, suggesting that neither of these terminal glycans are critical for the adhesion of *Corynebacterium* to sweat (data not shown).

Removal of *N*-glycans to assess any involvement in binding of *Corynebacterium* to sweat

The observation that sialidase and fucosidase treatment of sweat glycoproteins showed no effects on binding of *Corynebacterium* promoted the speculation that *N*-glycans are not involved at all in the binding interactions of these bacteria with sweat. To further investigate, PNGase F treatment was used to remove all *N*-glycans from the sweat proteins prior to testing bacterial binding in the plate assay. Before PNGase F treatment, the proteins were denatured at 95° C in the presence of SDS and DTT. Denaturation is required as standard protocol to allow access of PNGase F to glycans to increase deglycosylation efficiency. Therefore, binding was also compared to a similarly denatured Control (“Denatured Sweat Proteins without PNGase F”); and a “native” control (“Native Sweat Protein”) that received the same heat treatment at 95° C but in ammonium bicarbonate only, without SDS and DDT.

Figure 5: The binding of *Corynebacterium* species to sweat proteins was not reduced by PNGase F treatment, but significantly increased following denaturation and/or denaturation and PNGase F treatment of sweat proteins (* $p < 0.05$, t-test to No Sweat Protein). 1) Blocker only (No Sweat Protein); 2) Sweat proteins following heat treatment of proteins only (95° C, 5 min, Native Sweat Protein); 3) Denatured sweat proteins; 4) Denatured sweat proteins treated with PNGase F to remove *N*-glycans.

The binding of the *Corynebacterium* species to sweat proteins was not reduced by PNGase F treatment (Fig. 5), providing further evidence that *N*-glycans are not integral factors enabling sweat adhesion for these bacteria. In fact, the binding of *C. jeikeium* CIP103337 to de-*N*-glycosylated sweat proteins was statistically higher than to “native” sweat proteins and denatured sweat proteins without PNGase F treatment. We speculate that removal of *N*-glycans exposes binding counterparts for *C. jeikeium* on the protein backbone that are usually masked by *N*-glycans.

For *C. mucifaciens* 3-8BG-6, binding to the denatured sweat protein (both with and without PNGase F treatment) was much higher than to the “native” sweat protein (Fig. 5). The heat treatment applied to the “native” control in this experiment conceivably could have resulted in some structural changes that reduced binding affinity of *C. mucifaciens* 3-8BG-6 to sweat protein in comparison to previously observed (Fig. 1). Denaturation of the sweat proteins greatly increased binding of this bacterium to the sweat proteins (Fig. 5); we speculate that the protein denaturation has exposed more hydrophobic binding to the proteins by *C. mucifaciens* 3-8BG-6. Yet, there was no statistical difference between the binding of *C. mucifaciens* 3-8BG-6 to denatured sweat proteins treated with PNGase F compared to binding to denatured sweat proteins without PNGase F treatment, supporting the hypothesis that *N*-glycans are not integral to the binding of *Corynebacterium* to sweat.

Data from the above plate assays suggest *Corynebacterium* species do not use *N*-glycans on sweat glycoproteins for binding to sweat. In contrast, *N*-glycans do contribute to the binding of *S. epidermidis* to sweat, and there are strain-specific differences in preferred binding epitopes: both sialic acid and fucose may be potential binding partners of the *S. epidermidis* 3-22-BD-6, a strain recently isolated from human sweat; fucose (but not sialic acid) appears to be a significant contributor to the binding of the laboratory Type strain *S. epidermidis* ATCC 12228. Considering these results, we investigated whether synthetic glycans could inhibit the binding of *S. epidermidis* 3-22-BD-6 to sweat proteins; binding inhibition by specific sugars could potentially help confirm significant binding epitopes and act as possible inhibitors of bacterial binding to sweat.

Screening of potential inhibitors of *S. epidermidis* adhesion to sweat

A selection of synthetic glycan configurations representative of the Lewis A, B, X, Y and sialyl Lewis X blood group antigens (Elicityl, France; Fig. 6) were tested for their ability to inhibit the binding of clinical strain *S. epidermidis* 3-22-BD-6 to sweat proteins. The Lewis blood group antigens carry α 1-2 and/or α 1-3/ α 1-4 Fuc residues and are naturally generated throughout the human body and on secreted proteins according to individualised expression of glycosyltransferases

(Stanley and Cummings, 2017). Human pathogens have been found to bind to Lewis epitopes (Audfray *et al.*, 2012; de Mattos, 2016; Jeffries *et al.*, 2016; Saadi *et al.*, 1994), with strain specificities also reported (Chen *et al.*, 2011; Jin *et al.*, 2015). Since *S. epidermidis* 3-22-BD-6 was found to utilise both sialic acid and fucose in the above plate assays, we included sialyl Lewis X antigens amongst our range of potential inhibitors.

Figure 6: Glycan configurations of the Lewis blood group antigens used as potential inhibitors of bacterial adhesion to sweat (see *Inhibition assay*)

The results of the inhibition assays are shown in Figure 7. Pre-incubation of *S. epidermidis* 3-22-BD-6 with LeB, LeX, LeY and sLeX (pentaose) resulted in significantly reduced binding of the bacterium to the sweat proteins, and a similar but statistically insignificant trend was seen after incubation with LeA and sLeX (tetraose). The results indicate that terminal $\alpha 1-2$ fucosylation is a key binding epitope for *S. epidermidis* 3-22-BD-6 since incubation with all the sugars carrying terminal $\alpha 1-2$ fucosylation significantly inhibited binding to sweat. Furthermore, $\alpha 1-2$ fucosylation on a Type 2 chain (Gal $\beta 1-4$ GlcNAc) such as in the LeY epitope showed better inhibition activity compared to $\alpha 1-2$ fucosylation on a Type 1 chain (Gal $\beta 1-3$ GlcNAc) of LeB. LeX has no terminal $\alpha 1-2$ fucosylation but contains a Type 2 chain and showed inhibition. Thus, a Type 2 chain is also implicated as a potential binding epitope, secondary to $\alpha 1-2$ fucosylation. The sLeX (tetra) did not show inhibition activity but sLeX (penta) showed inhibition possibly due to the additional galactose on the reducing end of sLeX (penta) (Fig. 7).

Figure 7: Pre-incubation of *S. epidermidis* 3-22-BD-6 in synthetic glycan configurations representative of the LeB, LeX, LeY and sialyl LeX (penta) blood group antigens significantly inhibited adhesion of the bacteria to the sweat proteins (* $p < 0.05$, t-test to Sweat Protein).

This experiment provides a preliminary indication of the potential of glycans to inhibit the binding of *S. epidermidis* strains to sweat. Since recent reports emphasise the value of *S. epidermidis* as a protective commensal amongst the skin microbiota (Chen *et al.*, 2016; Nguyen *et al.*, 2017; Sullivan *et al.*, 2016), a glycan-related cosmetic that may reduce interaction of *S. epidermidis* with sweat without killing or removing the bacterium is particularly appealing. Conceivably the free inhibitory glycan mimics within a “deodorant” application could bind to *S. epidermidis* and keep them in the armpit in preference to the less desirable *Corynebacterium*, which appear not to use glycans as binding epitopes and are the most influential indicator of sweat-associated malodour (Callewaert *et al.*, 2017; Natsch, 2017). It is not yet known whether free glycans may also exist in sweat in addition to the protein-bound glycans studied here; irrespective, the possibility of designing competitive sugars to reduce interaction between sweat and odour-producing bacteria is a promising lead for future research and development in the cosmetic industry.

The fact that a *S. epidermidis* strain has been found to utilise fucose and sialic acid as binding epitopes, particularly $\alpha 1$ -2 fucosylation on a Type 2 chain (LeY) could also have implications in medical fields such as hospital settings where the bacterium is a leading cause of nosocomial infections on indwelling medical devices (Conlan *et al.*, 2012; Otto, 2014; Post *et al.*, 2017). The non-drug mode of action is particularly attractive considering the rise of antibiotic resistant and multidrug resistant *S. epidermidis* strains (Qin *et al.*, 2017; Widerstrom, 2016). Our work also confirms previous research indicating that strain variation exists (Chionh *et al.*, 2009; Kalograiaki *et*

al., 2016; Kautto *et al.*, 2016), so determining the preferred binding epitopes of each problematic strain would be of paramount importance.

Conclusion

This study represents an important stimulus for future research on the role of glycans in the adhesion of skin bacteria to sweat, with possible further cosmetic and/or therapeutic applications. We have discovered that the binding of two *S. epidermidis* strains to sweat proteins is *N*-glycan dependent, whereas the tested *Corynebacterium* strains appears not to be. Sugar mimics containing identified binding epitopes of a *S. epidermidis* strain have been demonstrated to reduce adhesion of the bacterium to sweat proteins. The present work may open new possible avenues for research addressing sweat-associated malodour reduction and, potentially, the fight against nosocomial infections.

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