J Membrane Biol (2015) 248:431–442
DOI 10.1007/s00232-015-9801-1

A Commensal Strain of *Staphylococcus epidermidis* Overexpresses Membrane Proteins Associated with Pathogenesis When Grown in Biofilms

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Received: 23 January 2015 / Accepted: 26 March 2015 / Published online: 3 April 2015

Abstract *Staphylococcus epidermidis* has emerged as one of the major nosocomial pathogens associated with infections of implanted medical devices. The most important factor in the pathogenesis of these infections is the formation of bacterial biofilms. Bacteria grown in biofilms are more resistant to antibiotics and to the immune defence system than planktonic bacteria. In these infections, the antimicrobial therapy usually fails and the removal of the biofilm-coated implanted device is the only effective solution. In this study, three proteomic approaches were performed to investigate membrane proteins associated to biofilm formation: (i) sample fractionation by gel electrophoresis, followed by isotopic labelling and LC–MS/MS analysis, (ii) in-solution sample preparation, followed by isotopic labelling and LC–MS/MS analysis and (iii) in-solution sample preparation and label-free LC–MS/MS analysis. We found that the commensal strain *S. epidermidis* CECT 231 grown in biofilms expressed higher levels of five membrane and membrane-associated proteins involved in pathogenesis: accumulation-associated protein, staphylococcal secretory antigen, signal transduction protein TRAP, ribonuclease Y and phenol soluble modulin beta 1 when compared with bacteria grown under planktonic conditions. These results indicate that a commensal strain can acquire a pathogenic phenotype depending on the mode of growth.

Keywords Membrane proteome · *Staphylococcus epidermidis* · Virulence · Biofilms

Introduction

Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix attached to inert or living surfaces (Costerton et al. 1999; Flemming and Wingender 2010). Biofilm formation occurs in response to a variety of environmental signals that lead to different phenotypes between attached cells and their planktonic (free swimming) counterparts. In particular, it has been described that bacteria in biofilms are 10- to 1000-fold more resistant to antibiotics than planktonic bacteria (Anwar and Costerton 1990; Arciola et al. 2005; Bjarnsholt et al. 2007). This fact is clinically relevant because bacterial biofilms cause chronic infections, including those associated to biomaterials (i.e., medical devices, prostheses, and catheters). In these cases, antibiotic treatment or the action of the host immune response is generally ineffective against the infection and removal of the implanted device is usually the only solution (Schwank et al. 1998; Mack et al. 2004).

Membrane proteins play an important role in biofilms, not only allowing the extrusion of toxic substances or antibiotics (Ehrlich et al. 2004), but also because they are responsible for essential physiological functions. Furthermore, about

Electronic supplementary material The online version of this article (doi:10.1007/s00232-015-9801-1) contains supplementary material, which is available to authorized users.

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one-third of the information in each mammalian genome encodes for membrane proteins that represent 60 % of the confirmed and putative drug targets (Hopkins and Groom 2002; Tan et al. 2008). These data and the fact that biological membranes form an essential barrier between living cells and their external environments suggest that membrane and membrane-associated proteins responsible for biofilm formation could be good therapeutic targets.

Staphylococcus aureus and *S. epidermidis* are the most frequent causative agents of nosocomial and biomaterial-associated infections in which surface-attached biofilms are involved (Hall-Stoodley et al. 2004; Sanchez et al. 2013). Often, *S. aureus* biofilm-associated infections are difficult to treat with antibiotics and devices need to be replaced more frequently than those infected with *S. epidermidis* (Jones et al. 2001). However, although *S. epidermidis* is a commensal bacterium on the human skin and mucous surfaces, it is known as an opportunistic pathogen that could cause an infection by penetration from the skin or mucous membranes after trauma, inoculation or implantation of medical devices (Arber et al. 1994). Comparative transcriptomic and proteomic studies between bacteria grown in biofilms and planktonic conditions have revealed that the cells in a biofilm have altered metabolic activity and gene expression differs between the two types of growth. These studies have been performed not only in staphylococcal strains, such as *S. aureus* (Resch et al. 2006; He and Ahn 2011) and *S. xylosus* (Planchon et al. 2009), but also in other species like *Bacillus cereus* (Oosthuizen et al. 2002), *Pseudomonas aeruginosa* (Steyn et al. 2001; Seyer et al. 2005), *Acinetobacter baumannii* (Siroy et al. 2006; Shin et al. 2009), *Salmonella typhimurium* (He and Ahn 2011) and *Neisseria meningitidis* (van Alen et al. 2010). Since proteins, not genes, are the active components in cells, the genome data do not provide direct information about the metabolic activity in biofilms. Proteomics should be a better tool to obtain data about the biological activity of the system (Yang et al. 2006). Thus, a comparative study at the proteomic level between biofilm and planktonic-grown bacteria would help in obtaining relevant information about differences between these two phenotypes.

In our study, we used the commensal strain *S. epidermidis* CECT 231. This strain which belongs to the risk group 1 according to the World Health Organization classification (WHO 2004) is able to grow in biofilms on glass wool using the method described by Steyn et al. (2001). Comparative membrane proteomic analyses were carried out using three different methods: (i) sample fractionation by gel electrophoresis, followed by isotopic labelling and LC–MS/MS analysis, (ii) in-solution sample preparation, followed by isotopic labelling and LC–MS/MS analysis and (iii) in-solution sample preparation and label-free LC–MS/MS analysis. Our investigation reveals that some membrane and membrane-associated proteins implied in virulence of *S. epidermidis* such as accumulation-associated protein, staphylococcal secretory antigen, signal transduction protein TRAP, ribonuclease Y and phenol soluble modulin beta 1 are overexpressed in biofilms. These results stress the risk of the mode of growth in commensal strains that can become virulent when grown in biofilms.

Materials and Methods

Cell Preculture

Staphylococcus epidermidis CECT 231, from the Colección Española de Cultivos Tipo (CECT), was used in all experiments. This strain belongs to the risk group 1 that according to the WHO is a microorganism that is unlikely to cause human disease or animal disease. Moreover, this strain also known as ATCC 12228 (from the American Type Collection Culture) is not associated with infections and it has been universally used in the last century for detection of residual antibiotics, e.g., gentamicin, neomycin, and novobiocin, in food products (Zhang et al. 2003). Cells were grown as described by Steyn et al. (2001) with some modifications. Preculture was performed in a 50-ml Erlenmeyer flask containing 10 ml of TSB medium incubated overnight at 37 °C with continuous shaking (200 rpm). Next, overnight culture was diluted (1:100) in 25 ml TSB, and the culture was incubated at 37 °C with continuous shaking (200 rpm) until mid-exponential phase ($OD_{540nm} = 0.1$). The culture was subsequently used to inoculate flasks for planktonic or biofilm biomass production.

Bacterial Growth Kinetics

To follow growth kinetics, OD_{540nm} values and colony-forming units (CFU) were measured. Biofilm growth kinetics were recorded as follows. Ten flasks containing 10 ml TSB supplemented with 0.1 % (w/v) glucose and 0.25 g sterile glass wool were inoculated at 4×10^5 CFU/ml. Bacteria were grown with shaking (200 rpm) at 37 °C for 30 h. Every 3 h, the glass wool from biofilm cultures was rinsed three times in 0.2 M sodium phosphate buffer (pH 6.8). Then it was placed in a sterile flask with 4.5 g glass beads (mean diameter 5 mm, Sigma-Aldrich) containing 20 ml 10 mM Tris–HCl buffer (pH 7.4), and bacterial biofilms were detached by shaking vigorously for 30 min. Bacteria were collected by centrifugation at 13,000×*g* for 10 min at 4 °C and resuspended in 10 ml 2 mM Tris–HCl buffer (pH 7.4), and 1 ml sample was taken to record the OD_{540nm}. After measuring OD_{540nm} values, appropriate dilutions of the cellular suspension were plated out on Tryptic

Soy Agar (Difco). Colonies were counted after plate incubation for 24 h at 37 °C. In like manner, planktonic growth kinetics were recorded from a culture inoculated with 4×10^5 CFU/ml as initial cell concentration by periodically measuring the OD_{540nm} and plating out appropriate dilutions of the cell suspension. Experiments were performed in triplicate.

Biomass Production for Proteomic Studies

Planktonic Cultures

For planktonic cultures, 4×10^7 CFU from the previously described preculture were inoculated in 100 ml TSB supplemented with 0.1 % (w/v) glucose. The culture was incubated at 37 °C for 11 h on a rotatory shaker at 200 rpm until the stationary phase was reached.

Biofilm Cultures

For biofilm cultures, 4×10^6 CFU from the preculture were inoculated in 30 flasks with 10 ml TSB supplemented with 0.1 % (w/v) glucose and containing 0.25 g glass wool (15–25 μm diameter, MERCK) for measuring bacteria attachment and biofilm formation. The flasks were incubated at 37 °C for 12 h in a rotatory shaker at 200 rpm until the stationary phase was reached.

Microscopy of Biofilm Development

Bright-Field Microscopy

To monitor biofilm development, glass wool was removed after 3, 6, 9, 12, 24 or 30 h growth, and then washed three times with distilled water. Subsequently, biofilms were stained with 0.1 % (w/v) crystal violet for 5 min at room temperature and washed three times with distilled water. Finally, biofilm formation was visualised with a Nikon Eclipse TS100 microscope (NY, USA). Images were captured with a Photometrics CoolSNAP cf camera (AZ, USA).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to perform a more detailed analysis of the biofilm structure covering the glass wool. The glass wool was removed after 12 or 30 h incubation and it was washed with distilled water to remove the planktonic bacteria. Then, samples were fixed in 2 % glutaraldehyde in 0.1 M Sorensen buffer (pH 7.4), washed in iso-osmolar Sorensen/sucrose buffer and fixed in 1 % (w/v) osmium tetroxide in Sorensen buffer. After repeated washes, samples were dehydrated with ethanol and washed in hexamethyldisilazane prior to air drying. Subsequently, samples were mounted onto stubs and gold coated. Finally, samples were visualised and micrographed using a scanning electron microscope (Hitachi S-3400) at 15 kV accelerating voltage.

Cell lysis and Membrane Preparation

Planktonic Cell Lysis

After incubation for 11 h at 37 °C, planktonic *S. epidermidis* cells were collected by centrifugation at $13{,}000 \times g$ for 10 min at 4 °C. The pellet was washed three times with 20 ml 0.2 M sodium phosphate buffer (pH 6.8) and then resuspended in 15 ml 2 mM Tris–HCl buffer (pH 7.4). Subsequently, cells were disrupted by sonication (10 s on/10 s off, 60 cycles) (Soniprep 150 MSE, UK). Afterwards, samples were centrifuged at $8000 \times g$ for 15 min at 4 °C. The pellet (unbroken cells) was discarded and the supernatant represented the cell lysate.

Biofilm Cell Lysis

The glass wool from biofilm cultures was removed after 12 h growth, rinsed three times in 0.2 M sodium phosphate buffer (pH 6.8) and placed in a sterile flask with 4.5 g glass beads (mean diameter 5 mm, Sigma-Aldrich). Then 20 ml 10 mM Tris–HCl buffer (pH 7.4) was added to the flask and it was shaken vigorously for 30 min to detach the bacterial biofilms from the glass wool surface. Bacteria were collected by centrifugation at $13{,}000 \times g$ for 10 min at 4 °C and then resuspended in 15 ml 2 mM Tris–HCl buffer (pH 7.4). Subsequently, cells were disrupted by sonication (10 s on/10 s off, 60 cycles) (Soniprep 150 MSE, UK). Next, samples were centrifuged at $8000 \times g$ for 15 min at 4 °C. The pellet (unbroken cells) was discarded and the supernatant represented the cell lysate.

Membrane Preparation

In order to obtain the membrane protein-enriched fraction, cell lysates were treated as previously described (Molloy et al. 2000) with modifications (Soares et al. 2009). Briefly, cell lysate was diluted with an equal volume of 0.1 M Na_2CO_3 (pH 11) and the mixture was stirred slowly overnight at 4 °C. Next, the mixture was ultracentrifuged at $115{,}000 \times g$ for 1 h at 4 °C. The supernatant was discarded, the membrane pellet was resuspended in 8 ml 2 mM Tris–HCl (pH 7.4) and a new pellet was collected by ultracentrifugation at $115{,}000 \times g$ for 1 h at 4 °C. Finally, the pellet corresponding to the membrane protein-enriched fraction was resuspended in 70 μl of 2 mM Tris–HCl (pH 7.4) and stored at −20 °C.

Protein Quantification

Protein concentration was determined by BioRad commercial kit (BCA Protein Assay kit) following manufacturer's instructions.

Sample Preparation and Isotopic Labelling for Mass Spectrometry

In-Gel Sample Preparation

Samples (20 μg membrane protein-enriched fraction) were reduced with 5 mM tris(2-carboxyethyl)phosphine, alkylated with 15 mM iodoacetamide and fractionated by SDS-PAGE on 10–20 % acrylamide gradient gels (Life Technologies) in Tris–glycine buffer. Gel sections were excised and diced, and proteins were digested in-gel with trypsin (12.5 ng/μl) (Shevchenko et al. 1996).

In-Solution Sample Preparation

Samples were processed as previously described (Carroll et al. 2006) with some modifications. Briefly, a volume corresponding to 100 μg membrane protein-enriched fraction (defined as 1 vol) was vortexed occasionally for 5 min at room temperature in 9 vol of a mixture of chloroform and methanol in 2 mM Tris–HCl (pH 7.4) [66.7:31.3:2 (v/v/v)]. The aqueous and hydrophobic phases were discarded. The interphase corresponding to protein precipitation was washed with methanol and centrifuged at 16,000×*g* for 5 min at room temperature. The pellet was reduced with 5 mM tris(2-carboxyethyl)phosphine and alkylated with 15 mM iodoacetamide in 100 μl of 50 mM Tris–HCl (pH 8) buffer containing 6 M guanidinium chloride and 1 mM EDTA. Then, the samples were dialyzed overnight against 100 mM ammonium bicarbonate (NH_4HCO_3) using Slide-A-Dialysis Cassettes (Thermo Scientific). In-solution tryptic digestion was carried out in 100 μl of 100 mM NH_4HCO_3 for 18 h at 37 °C with agitation. The ratio of trypsin to sample was 1:50 (w/w). The samples were dried by vacuum centrifugation and stored at −20 °C until the isotopic labelling was performed.

For the label-free LC–MS/MS analysis, 50 μg membrane protein-enriched fraction was digested following FASP protocol (Wisniewski et al. 2009) and digested peptides were desalted and concentrated using C18 Micro Spin Columns (Harvard Apparatus, Holliston, MA, USA).

In-Solution Isotopic Labelling

Isotopic labelling was carried out as described by Boersema et al. (2009). Digested samples (<25 μg of protein) were reconstituted in 100 μl of 100 mM triethyl ammonium bicarbonate (TEAB). Biofilm and planktonic samples were differentially labelled (light and heavy isotopes) in parallel in two different tubes. 4 μl of 4 % (v/v) formaldehyde (CH_2O) or deuterium formaldehyde (CD_2O) was added to the sample to be labelled with light and heavy dimethylation, respectively. The samples were mixed briefly and the solution spun down. 4 μl of 600 mM sodium cyanoborohydride ($NaBH_3CN$) was added to the samples and they were incubated in a fume hood for 1 h at room temperature (15–22 °C) while mixing using a bench top test tube mixer. The labelling reaction was quenched by adding 16 μl of 1 % (v/v) ammonia solution, it was mixed briefly and then the solution was spun down. 8 μl of 5 % (v/v) formic acid in water was added to further quench the reaction and to acidify the sample for subsequent LC–MS analysis. The differentially labelled samples were mixed and dried by vacuum centrifugation. The samples were stored at −20 °C.

Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS) and Data Analysis

The differentially labelled samples were mixed and resuspended in 5 % (v/v) aqueous acetonitrile containing 0.1 % (v/v) formic acid. Peptides were fractionated in a Proxeon Easy-nLC system on a nanoscale reverse-phase column (75-μm inner diameter, 100-mm long; Nanoseparations, Nieuwkoop, Netherlands). A gradient (0–40 %) of 95 % (v/v) aqueous acetonitrile which contains 0.1 % (v/v) formic acid over 84 min with a flow rate of 300 nl/min was used. The effluent was passed directly into an LTQ OrbiTrap XL mass spectrometer (Thermo Fisher, Hemel Hempstead, HP27GE, UK) operating in data-dependent MS/MS mode, with a mass scan range of 400–2000 Da for precursor ions and MS/MS of the top 10 highest abundance ions selected from the full scan. Isotope-labelled peptide pairs were located by MaxQuant and identified using the Mascot algorithm with the *S. epidermidis* (strain ATCC 35984/RP62A) UniProtKB database.

Label-free LC–MSE analysis was performed in a SYNAPT HDMS mass spectrometer (Waters) interfaced with a NanoAcquity UPLC System (Waters, Milford, MA, USA). 4 μl containing 1 μg of protein and 100 fmol of MassPREP Enolase Digestion Standard were loaded onto a Symmetry 300 C18, 180 μm × 20 mm precolumn (Waters, Milford, MA, USA). The precolumn was connected to a BEH130 C18, 75 μm × 200 mm, 1.7 μm (Waters), and peptides were eluted with a 120 min linear gradient from 3 to 40 % of acetonitrile followed by a 15 min linear gradient from 40 to 60 % of acetonitrile. Mass spectra were acquired using a data-independent acquisition mode (MSE) described by Silva et al. (2005). Briefly, 1 s alternate MS acquisitions were performed at low (6 eV) and high (12–35 eV ramping) collision energies, and the RF (radio frequency) offset was adjusted

such that the MS data were acquired from *m/z* 350 to 1990. Acquired spectra were processed with ProteinLynx Global Server 2.4 Build RC7 (Waters, Milford, MA, USA). Protein identification was obtained with the embedded database search algorithm of the programme (Li et al. 2009), and a *S. epidermidis* (strain ATCC 35984/RP62A) UniProtKB database (version 2013_06), where the ENO1_YEAST and TRYP_PIG sequences were added, was used. Absolute protein quantification was performed comparing the sum of the intensities of the three most intense peptides of the standard protein (100 fmol of MassPREP Enolase Digestion Standard per sample) versus the sum of the intensities of the three most intense peptides of each of the identified proteins (Silva et al. 2006). Therefore, only proteins identified with at least three peptides were used for absolute quantification. Protein quantification was normalized against the total amount of protein per injection, and additional statistical analysis was performed with Microsoft Excel (Microsoft, Redmond, WA, USA).

Detection of the *icaAB* Gene Complex by PCR

The presence of *icaAB* genes was analysed by PCR using oligonucleotide primers, icaAB-F: 5′-TTATCAATGCCG CAGTTGTC-3′ (forward) and icaAB-R: 5′-GTTTAACGC GAGTGCGCTAT-3′ (reverse) from partial *icaAB* genes of *S. epidermidis* previously described (Martin-Lopez et al. 2004). PCR assays were performed as follows. Each 25 µl PCR mixture contained 1.5 U *Taq* DNA polymerase (New England Biolabs), 1× Standard *Taq* reaction buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 0.5 µM of each primer, 200 µM dNTPs and 2 µl of template DNA. As template DNA, *S. epidermidis* cell lysates were used. For the cell lysis, an overnight culture was centrifuged at 11,000×*g* for 1 min, and the pellet was suspended in 20 µl lysis buffer [50 mM NaOH, 0.25 % (v/v) SDS]. 2 µl 1:10 dilution of cell lysate was used as DNA template. As a negative control, all the PCR reagents without DNA were used. DNA was amplified in a C1000™ Thermal Cycler (Bio-Rad) with the following thermal profile: an initial denaturation step at 94 °C for 5 min was followed by 30 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s), ending with a final extension step at 72 °C for 5 min. After PCR amplification, 10 µl of PCR product was resolved by 1 % (w/v) agarose gel electrophoresis and visualised by staining with SYBR® Safe DNA Gel Stain (Invitrogen).

Western Blotting of Accumulation-Associated Protein (Aap)

Triplicate planktonic or biofilm biological samples were used for Aap detection. Thus, 5 µg of each membrane protein-enriched fraction of *S. epidermidis* CECT 231 planktonic and biofilm samples was separated electrophoretically on 10 % SDS–polyacrylamide gels and transferred to nitrocellulose membrane. The transfer was performed using 15 V for 1 h in transfer buffer [2.9 g/l glycine, 5.8 g/l Tris, 3.75 % (w/v) SDS and 20 % (v/v) methanol]. In order to avoid non-specific bindings, nitrocellulose membrane was blocked for 1 h at room temperature with 10 % (w/v) skim milk in Tris-buffered saline solution containing 0.1 % Tween 20 (pH 7.6) (TBST). The blocking solution was removed, and nitrocellulose membrane was incubated overnight with the primary anti-Aap antibody (kindly provided by Professor H. Rohde) diluted (1:20,000) in TBST containing 5 % (w/v) skim milk at 4 °C. After three washes with TBST, the membrane was incubated for 1 h at room temperature with secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) (1:4000) in TBST containing 5 % (w/v) skim milk. After three washes with TBST, bands were visualised by enhanced chemiluminescence (Thermo Scientific) and exposed films were analysed by Quantity One software.

Results

S. epidermidis CECT 231 Cells formed Biofilms on Glass Wool

Planktonic and biofilm growth of *S. epidermidis* CECT 231 was followed by two different methods. CFU/ml of planktonic and biofilm cultures were measured (Fig. 1a). Also OD_{540nm} values were recorded in order to obtain planktonic and biofilm growth curves (Fig. 1b). Figure 1a shows that planktonic cultures of *S. epidermidis* CECT 231 reached the stationary phase after 11 h growth and that the cell concentration at that point was 10^9 CFU/ml. Biofilm cultures reached the stationary phase after 12 h growth, and cell concentration was 10^8 CFU/ml. Similarly, when OD_{540nm} values were measured, it was observed that planktonic and biofilm cultures reached stationary phase after 11 and 12 h growth, respectively (Fig. 1b). Biomass and membrane protein extraction for subsequent studies were achieved from biofilm and planktonic cultures at stationary phase.

Biofilm formation by *S. epidermidis* CECT 231 cells on glass wool was monitored by bright-field microscopy. The culture medium was TSB supplemented with 0.1 % (w/v) glucose, and images were taken at 3, 6, 9, 12, 24 and 30 h growth. As shown in Fig. 2 (white arrow), microcolonies were formed after 9 h growth, and dense biofilm structures were observed after 12, 24 and 30 h growth.

SEM images show differences in the morphology of biofilms grown at different times. In particular, biofilms formed after 12 h growth showed an extracellular matrix covering the cells where individual cells could not be easily distinguished. In contrast, in biofilms formed after 30 h

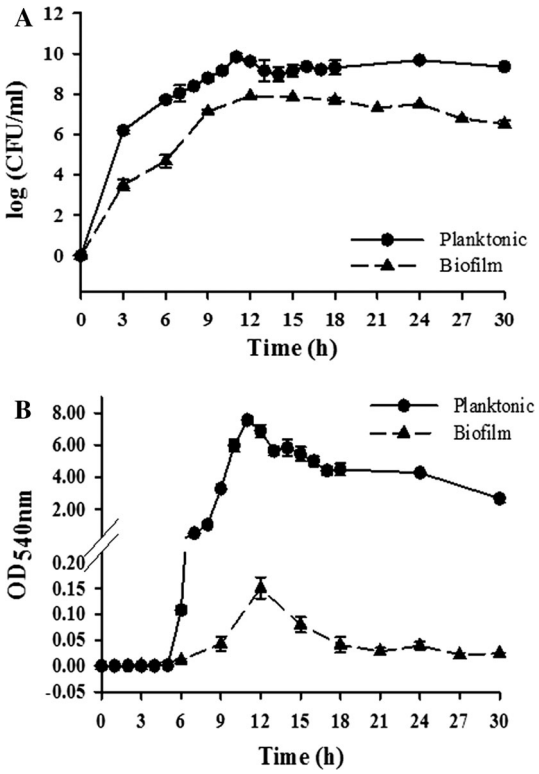

Fig. 1 Planktonic and biofilm growth curves of *S. epidermidis* CECT 231. Bacteria were inoculated in TSB medium supplemented with 0.1 % (w/v) glucose and grown at 37 °C with shaking. Growth curves were obtained by counting the colony forming units per millilitre (CFU/ml) (**a**) and by measuring the OD_{540nm} values (**b**)

growth, individual cells attached to the glass wool that could leave the biofilm and propagate infection were observed (Fig. 3).

S. epidermidis CECT 231 Proteome Revealed that Virulence Proteins were More Abundant in Biofilms than in Planktonic Cultures

Membrane-enriched fractions of three independent biological samples of planktonic (P1, P2, P3) and biofilm (B1, B2, B3) cultures at stationary state were compared after separation by SDS-PAGE. No significant differences between the protein profiles of both growth conditions were observed (data not shown). Therefore, in order to further analyse membrane and membrane-associated protein expression differences among planktonic and biofilm cultures, three different proteomic methods were performed: (i) sample fractionation by gel electrophoresis, followed by isotopic labelling and LC–MS/MS analysis, (ii) in-solution sample preparation, followed by isotopic labelling and LC–MS/MS analysis and (iii) in-solution sample preparation and label-free LC–MS/MS analysis.

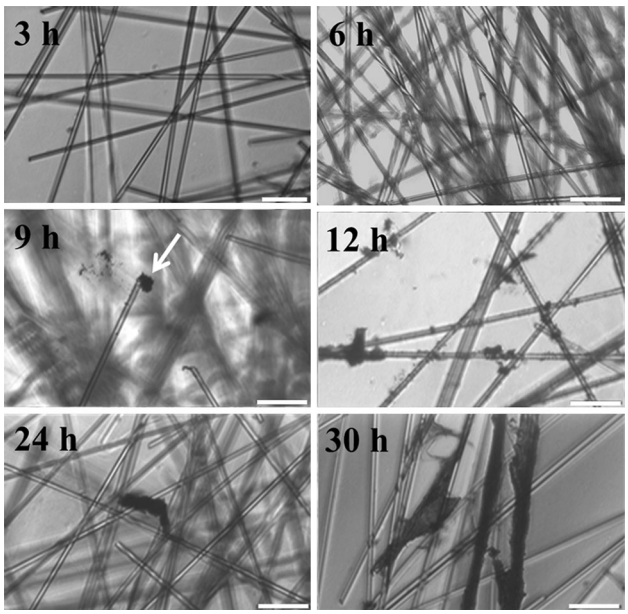

Fig. 2 Bright-field microscopy images of *S. epidermidis* CECT 231 biofilms grown on glass wool. Biofilms were grown on glass wool and collected after 3, 6, 9, 12, 24 and 30 h. Then biofilms were stained with crystal violet 0.1 % (w/v) as described in "Materials and Methods" section. *Scale bars* represent 100 µm. *White arrow* indicates the initial stages of biofilm formation

Sample Fractionation by Gel Electrophoresis, Followed by Isotopic Labelling and LC–MS/MS Analysis

Equal amounts of membrane protein-enriched fractions from planktonic and biofilm cultures were resolved by gel electrophoresis. Each lane was divided into ten pieces that were excised and proteolysed in-gel with trypsin (Fig. 4). After protein digestion, the planktonic and biofilm peptides were individually labelled with either heavy or light isotopes using the dimethyl labelling method as described in "Materials and Methods" section. Duplicate labelling experiments were performed as follows: in the first experiment, the biofilm sample was labelled with the heavy isotope and the planktonic sample with the light isotope. This experiment was named "heavy biofilm (HB)". In the second experiment, the biofilm sample was labelled with the light isotope, while the planktonic sample was labelled with the heavy isotope. This experiment was named "light biofilm (LB)".

This method identified 578 proteins in planktonic and biofilm growth conditions. Biologically significant expression differences were considered when variations between planktonic and biofilm conditions were more than twofold. The biofilm proteome showed 34 proteins expressed more than twofold in comparison to planktonic cultures in duplicate experiments. Nevertheless, according to the significance B values in the MaxQuant programme, only the staphylococcal secretory antigen (SsaA) (Q5HLV2) was statistically

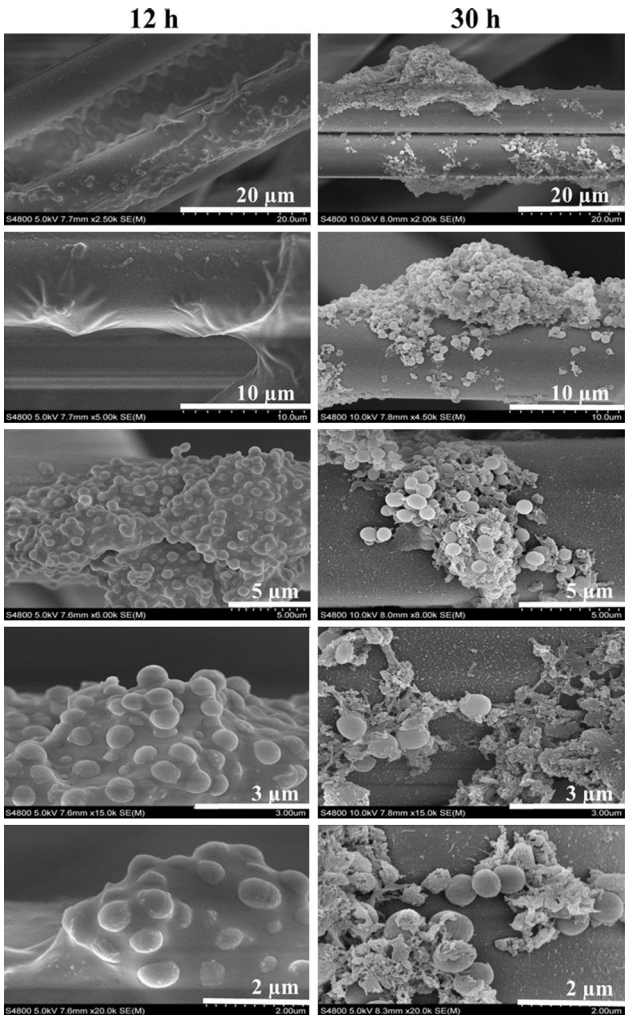

Fig. 3 Scanning electron micrographs of *S. epidermidis* CECT 231 biofilms after 12 h (*left column*) and 30 h (*right column*) of growth. Biofilms were grown on glass wool for 12 and 30 h. Samples were processed and coated with gold as described in "Materials and Methods" section. SEM images were taken at different magnifications as indicated in the panels

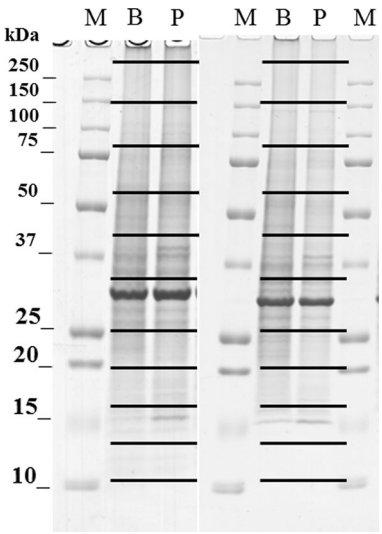

Fig. 4 In-gel fractionation of membrane protein-associated fractions from *S. epidermidis* CECT 231. Membrane protein-associated fractions of *S. epidermidis* CECT 231 grown under planktonic (*P*) and biofilm (*B*) conditions were electrophoresed on 10–20 % acrylamide gradient SDS-PAGE gels. 20 µg protein was loaded into each lane. Fractionation into ten pieces was carried out from each lane for further proteomic analysis. *Lane M* molecular weight markers

significant in both labelling experiments. Both proline dehydrogenase (Q5HNE5) and putative aldehyde dehydrogenase (AldA) (Q5HLA3) proteins were also statistically significant, but only in one of the labelling experiments. The results of the analysis of biofilm cultures are summarized in Supplementary Table S1.

In planktonic growth conditions, it was observed that 19 proteins were expressed more than twofold in comparison to the biofilm condition in both labelling experiments. Again in this case only one protein, formate acetyltransferase (Q5HKH9), was statistically significant in duplicate labelling experiments. In this study, two proteins [oxidoreductase, short-chain dehydrogenase/reductase family (Q5HKT5) and anaerobic ribonucleoside-triphosphate reductase (Q5HL04)] were statistically significant only in one of the labelling experiments. The planktonic proteome is summarized in Supplementary Table S2.

Fractionation from SDS-PAGE gel followed by dimethyl labelling is a good technique to identify and quantify differentially expressed proteins between two conditions. Nevertheless, its complexity in terms of number of samples and sample manipulation steps compromises its reproducibility. To overcome these limitations, we carried out two other approaches in which the samples were not fractionated by SDS-PAGE gels before LC–MS/MS analysis. In both cases, samples were trypsin digested in solution. In one case, membrane protein-enriched fractions were quantified by isotopic dimethyl labelling, and in the other by label-free LC–MS/MS analysis.

In-Solution Sample Preparation, Followed by Isotopic Labelling and LC–MS/MS Analysis

Two different biological samples (biofilm and planktonic) and two biochemical replicas with reciprocal isotope labelling for each biological sample were used in this study. The proteins expressed more than twofold in biofilm over planktonic conditions are represented in Supplementary Table S3. Those expressed more than twofold in planktonic over biofilm conditions are shown in Supplementary Table S4. In the case of biofilm proteome, five proteins were expressed more than twofold in biofilm condition in comparison to planktonic growth, in at least one duplicate labelling experiment (Supplementary Table S3). One of these

five proteins, the 50S ribosomal protein L21 (Q5HNQ4), was statistically significant in both labelling experiments from one biological sample. Aap (Q5HKE8) and glycerol kinase (Q5HPP1) were also statistically significant but only in one of the duplicate labelling experiments from one biological sample.

In the planktonic state, it was observed that 15 proteins were expressed more than twofold in comparison to biofilm growth (Supplementary Table S4). Nevertheless, only the ABC transporter substrate-binding protein (Q5HRA3) was statistically significant in one biological sample, but this significance was present in both duplicate labelling experiments.

In-Solution Sample Preparation and Label-Free LC–MS/MS Analysis

Three different biological samples of biofilm and planktonic conditions were analysed. This method identified 424 proteins in planktonic and biofilm growth conditions. Protein relative quantification was only calculated for proteins identified at least in two replicates with a minimum of three peptides in each growth condition. Those proteins with expression ratios above 2 and a Student's *t* test value below 0.05 were only considered as significant. The most abundant proteins found in biofilm and planktonic conditions are summarized in Supplementary Tables S5 and S6, respectively. It was observed that in biofilms, two proteins were expressed more than twofold in comparison to planktonic cells with statistical significance. Under planktonic conditions, eight proteins were expressed more than twofold with statistical significance as compared to biofilms. However, considering proteins identified only in one of the two conditions, it was also observed that the phenol soluble modulin beta 1 protein (Q5HQ19) was only identified in the three biological replicates of the biofilm samples. This suggests a differential expression of this protein in biofilm and planktonic samples. To further verify this result, the precursor ions of the identified peptides (high confidence and Pass One Match Peptides in ProteinLynx Global Server) were manually inspected. The extracted ion chromatograms of the precursor ions were quantified, and a minimum threefold expression ratio was determined (data not shown).

In an attempt to show the overexpressed and statistically significant proteins found in these three proteomic approaches, Tables 1 and 2 were prepared. Table 1 summarizes the statistically significant and more abundant proteins in biofilm mode of growth according to at least one of the three proteomic methods employed. Correspondingly, Table 2 summarizes the statistically significant and more abundant proteins in planktonic condition found by at least one of the three proteomic methods employed.

S. epidermidis CECT 231 is an *icaADBC*-Negative Staphylococcal Strain that Forms Biofilms

The polysaccharide intercellular adhesin (PIA), encoded by the *icaADBC* operon, is one of the major factors involved in biofilm formation. PIA expression has been described as an important process for the pathogenesis of *S. epidermidis* biomaterial-related infections (Rupp et al. 1999a, b; Szczuka and Kaznowski 2014). However, it has also been demonstrated that *icaADBC*-negative staphylococcal strains are able to form biofilms and cause this type of infections (Rohde et al. 2005; Stevens et al. 2009; Liduma et al. 2012; Szczuka et al. 2013). In this work, the presence of *icaAB* gene complex was analysed in a lysate of the commensal strain *S. epidermidis* CECT 231 by PCR. In Fig. 5, it can be observed that *icaAB* gene complex is not

Table 1 Overexpressed proteins in *S. epidermidis* CECT 231 biofilms, identified by at least one of the three proteomic methods used in this work

Uniprot	Protein description	Proteomic method		
		Gel-fractionation and dimethyl labelling	In-solution treated sample and dimethyl labelling	In-solution treated sample without labelling
Q5HLV2	Staphylococcal secretory antigen (SsaA)	X		
Q5HNE5	Proline dehydrogenase	X		
Q5HLA3	Putative aldehyde dehydrogenase (AldA)	X		
Q5HNQ4	50S ribosomal protein L21		X	
Q5HKE8	Accumulation-associated protein (Aap)		X	
Q5HPP1	Glycerol kinase		X	
Q5HNH	Formate-tetrahydrofolate ligase			X
Q5HPU5	Succinyl-CoA ligase [ADP-forming] subunit beta			X
Q5HQ19	Phenol soluble modulin beta 1			X

Only statistically significant proteins are listed. *X* proteins quantified using the corresponding proteomic method

Table 2 Overexpressed proteins in planktonic *S. epidermidis* CECT 231, identified in at least one of the three proteomic methods described in this work

Uniprot	Protein description	Proteomic method		
		Gel-fractionation and dimethyl labelling	In-solution treated sample and dimethyl labelling	In-solution treated sample without labelling
Q5HKH9	Formate acetyltransferase	X		
Q5HKT5	Oxidoreductase, short-chain dehydrogenase/reductase family	X		
Q5HL04	Anaerobic ribonucleoside-triphosphate reductase	X		
Q5HRA3	ABC transporter, substrate-binding protein		X	X
Q5HR34	PTS system, fructose-specific IIABC components			X
Q5HR68	Flavohemoprotein, putative			X
Q5HL31	L-Lactate dehydrogenase			X
Q5HQA9	Probable quinol oxidase subunit 2			X
Q5HN34	Glutamyl-tRNA(Gln) amidotransferase subunit A			X
Q5HM04	50S ribosomal protein L22			X
Q5HLT9	Transcriptional regulator, putative			X

Only statistically significant proteins are listed. *X* proteins quantified using the corresponding proteomic method

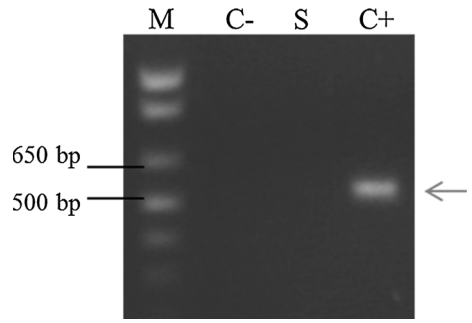

Fig. 5 *icaAB* gene detection in *S. epidermidis* CECT 231 by PCR and agarose gel analysis. *icaAB* amplicons (546 bp) obtained after PCRs were analysed on 1 % (w/v) agarose gel. *Lane C−* negative control; *lane S icaAB* amplicon from *S. epidermidis* CECT 231; *lane C+* positive control, *icaAB* amplicon from *S. epidermidis* clinical isolate 338515-1. *Lane M* DNA molecular weight marker 1 kb Plus DNA ladder. *Black arrow* shows a positive amplicon for *icaAB* gene

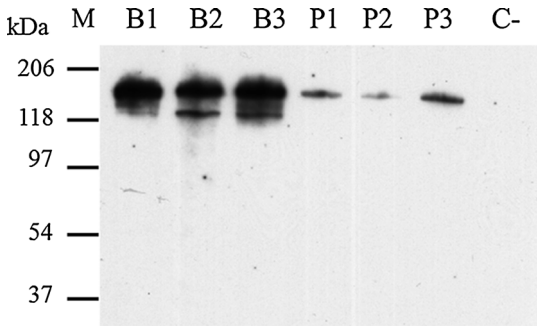

Fig. 6 Expression of Aap in *S. epidermidis* CECT 231 grown in biofilm and planktonic conditions. 5 μg of membrane protein-associated fraction was loaded on SDS-PAGE gel, and western blotting assay was carried out using anti-Aap antiserum. *B1*, *B2* and *B3* biofilm biological samples; *P1*, *P2* and *P3* planktonic biological samples; *C−* 5 μg of bovine serum albumin (BSA); *M* molecular weight marker

present in this strain. Nevertheless, it has been shown that *S. epidermidis* CECT 231 is able to grow under biofilm conditions.

Aap, a Protein Involved in Virulence, is Up-Regulated in Biofilm Growth Condition

Our proteomic studies have shown that Aap is one of the proteins overexpressed by *S. epidermidis* CECT 231 grown in biofilms. To confirm the overexpression of Aap in biofilms of this commensal strain, a Western blotting with anti-Aap antiserum was carried out. Three biofilm and planktonic biological samples were used for that purpose. The same amount of membrane protein-enriched fractions from these six samples was loaded on SDS-PAGE gel. Figure 6 shows a band between 206 and 118 kDa that was present in all the samples. When biofilm and planktonic conditions were compared, the Western blotting assay showed significantly higher amount of Aap in the three biofilm samples in comparison to the planktonic ones (Fig. 6). This band can be assigned to the 180 kDa Aap isoform described previously (Rohde et al. 2005). More interestingly, an additional band corresponding to the 140 kDa Aap active isoform was also observed in biofilm samples only.

Discussion

Glass wool provides a large surface-to-volume ratio and allows the convenient collection of the adherent bacterial biomass. In this work, the commensal strain *S. epidermidis* CECT 231 was grown to form biofilms on glass wool, and the biofilm biomass obtained by this method after 12 h growth was sufficient for subsequent proteomic analyses. To study the development of biofilms grown on glass wool, bright-field microscopy was used. The first microcolonies appeared after 9 h bacterial incubation, and larger biofilms were observed at longer times (12, 24 and 30 h). Similar formation rates and morphologies of *P. aeruginosa* and *B. cereus* biofilms have been shown by bright-field microscopy by other authors (Steyn et al. 2001; Oosthuizen et al. 2002). These bright-field microscopy experiments allowed us to determine biofilm formation for different periods of time and to observe the distribution of cell clusters over the surface and their size, but details of their structure could not be distinguished. Thus, we carried out SEM studies of biofilm morphology after 12 and 30 h growth. These images showed that after 12 h growth, cells were embedded in the extracellular matrix. In contrast, this matrix was not observed after 30 h of biofilm growth (Fig. 3) which suggests that the community gave rise to non-sessile bacteria, planktonic cells that can rapidly multiply and disperse (Costerton et al. 1999).

It is well known that bacteria grown in biofilm express different genes as compared to their planktonic counterparts. These data have been revealed by many comparative proteomic studies between both growth conditions in several species, such as *S. aureus* (Resch et al. 2006; He and Ahn 2011), *S. xylosus* (Planchon et al. 2009), *B. cereus* (Oosthuizen et al. 2002), *P. aeruginosa* (Steyn et al. 2001; Seyer et al. 2005), *A. baumannii* (Siroy et al. 2006; Shin et al. 2009), *S. typhimurium* (He and Ahn 2011) and *N. meningitidis* (van Alen et al. 2010). In particular, a comparative proteomic analysis between invasive and commensal strains of *S. epidermidis* has been reported by Yang et al. (2006). Nevertheless, hitherto, no comparative proteomic analysis between the same *S. epidermidis* strain grown in biofilm versus planktonic state has been reported. Our study compares membrane-associated proteome between the commensal strain *S. epidermidis* CECT 231 grown under biofilm and planktonic conditions.

Among the biofilm up-regulated proteins, we found membrane and membrane-associated proteins related to infections caused by staphylococcal biofilms. In addition to SsaA and Aap, the virulence proteins ribonuclease Y, signal transduction protein TRAP and the phenol soluble modulin beta 1 were more abundant in biofilm cultures. Yang et al. (2006) observed higher levels of Aap and protein TRAP in an invasive *S. epidermidis* strain when compared to commensal one by proteomic analysis. Moreover, a comparative proteomic analysis of *S. aureus* biofilm and planktonic cells showed that an Aap homolog protein was found in higher amount in biofilms in comparison to planktonic cells (Resch et al. 2006). Regarding virulence proteins ribonuclease Y and phenol soluble modulin beta 1, this is the first time that they have been detected by proteomic analysis as more abundant in biofilms when compared to planktonic cells.

On one hand, SsaA has been found in biofilm-associated infections where anti-SsaA immunoglobulin G antibody levels were higher in sera of patients with *S. epidermidis* endocarditis (Lang et al. 2000). Moreover, the expression level of *ssaA* gene in *S. aureus* biofilms after 16 h growth was higher than its expression under planktonic growth conditions (Resch et al. 2005). Additionally, a secretomic analysis done by Visutthi et al. (2011) in methicillin-resistant *S. aureus* (MRSA) revealed that SsaA was down-regulated after a treatment with rhodomyrtone. Nevertheless, the specific role of this protein seems to be more related to the pathogenesis of *S. epidermidis* infections than to the biofilm formation process (Lang et al. 2000).

On the other hand, Aap has been considered to be essential for bacterial accumulation in biofilm formation and it has been proposed as a novel immunotherapeutic target for prevention of foreign body-related infections (Schumacher-Perdreau et al. 1994; Hussain et al. 1997; Yang et al. 2006). This protein has been proposed as a target for anti-biofilm vaccines, and different monoclonal antibodies against Aap have been generated (Sun et al. 2005; Hu et al. 2011; Yan et al. 2014). In this regard, although some monoclonal antibodies could reduce and even eliminate *S. epidermidis* bacterial colonization on biomedical devices, there are not conclusive results. Moreover, the proteolytic processing of Aap has been confirmed as a PIA-independent mechanism of biofilm formation (Rohde et al. 2005). In agreement to these authors, our results show that the proteolysed 140 kDa isoform is only present in the biofilm samples, whereas the planktonic samples only contain the inactive isoform of Aap (Fig. 6). This truncated 140 kDa isoform has been previously described to be responsible for the activity of Aap in biofilm formation by intercellular adhesion in a polysaccharide-independent manner (Rohde et al. 2005). This membrane protein is currently being studied for the development of anti-biofilm vaccines (Yan et al. 2014) and it could also be considered as a good therapeutic target, especially in those strains that do not contain the *ica* operon.

In conclusion, this work shows that even in a commensal *S. epidermidis* strain, membrane and membrane-associated proteins involved in virulence could be expressed under biofilm growth, stressing the risk of the

biofilm mode of growth even in commensal strains. These proteins, in particular Aap, could be good therapeutic targets to control biofilm infections. Hence, therapeutic strategies that could inhibit the expression or activity of these genes and their products in *S. epidermidis* biofilm formation are likely to provide novel potentially beneficial alternatives to current therapies.

Acknowledgments S.A-A thanks the Basque Government and the Medical Research Council for supporting her stage in Cambridge. We also thank Eneritz Bilbao and Joe Carroll for their excellent technical assistance. The authors acknowledge the Analytical and High-Resolution Microscopy in Biomedicine Service at the University of Basque Country for assistance with Scanning Electron Microscopy. The Proteomics Core Facility-SGIKER (member of ProteoRed-ISCIII) is supported by Universidad del País Vasco/Euskal Herriko Unibertsitatea, Ministerio de Ciencia e Innovación, Gobierno Vasco/Eusko Jaurlaritza, and European Science Foundation. This work was supported by funds from the Spanish Ministry of Economy (Grant No. BFU2012-36241) and from MICINN (Grant No. BFU2010-22103). S.A-A was a pre-doctoral student supported by the Basque Government and by the Fundación Biofísica Bizkaia. We thank Prof. Holger Rohde from the Institut für Medizinische Mikrobiologie, Virologie und Hygiene (Hamburg, Germany) for supplying the anti-Aap antiserum.

References

Anwar H, Costerton JW (1990) Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 34:1666–1671

Arber N, Pras E, Copperman Y, Schapiro JM, Meiner V, Lossos IS, Militianu A, Hassin D, Pras E, Shai A (1994) Pacemaker endocarditis. Report of 44 cases and review of the literature. Medicine (Baltimore) 73:299–305

Arciola CR, Campoccia D, Gamberini S, Donati ME, Pirini V, Visai L, Speziale P, Montanaro L (2005) Antibiotic resistance in exopolysaccharide-forming *Staphylococcus epidermidis* clinical isolates from orthopaedic implant infections. Biomaterials 26:6530–6535

Bjarnsholt T, Kirketerp-Moller K, Kristiansen S, Phipps R, Nielsen AK, Jensen PO, Hoiby N, Givskov M (2007) Silver against *Pseudomonas aeruginosa* biofilms. APMIS 115:921–928

Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat Protoc 4:484–494

Carroll J, Fearnley IM, Walker JE (2006) Definition of the mitochondrial proteome by measurement of molecular masses of membrane proteins. Proc Natl Acad Sci USA 103:16170–16175

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Ehrlich GD, Hu FZ, Post JC (2004) Role for biofilms in infectious disease. In: Ghannoum M, O'Toole GA (eds) Microbial biofilms. ASM Press, Washington, DC, pp 332–358

Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623–633

Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2:95–108

He X, Ahn J (2011) Differential gene expression in planktonic and biofilm cells of multiple antibiotic-resistant *Salmonella typhimurium* and *Staphylococcus aureus*. FEMS Microbiol Lett 325:180–188

Hopkins AL, Groom CR (2002) The druggable genome. Nat Rev Drug Discov 1:727–730

Hu J, Xu T, Zhu T et al (2011) Monoclonal antibodies against accumulation-associated protein affect EPS biosynthesis and enhance bacterial accumulation of *Staphylococcus epidermidis*. PLoS ONE 6:e20918

Hussain M, Herrmann M, von Eiff C, Perdreau-Remington F, Peters G (1997) A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. Infect Immun 65:519–524

Jones SM, Morgan M, Humphrey TJ, Lappin-Scott H (2001) Effect of vancomycin and rifampicin on meticillin-resistant *Staphylococcus aureus* biofilms. Lancet 357:40–41

Lang S, Livesley MA, Lambert PA, Littler WA, Elliott TS (2000) Identification of a novel antigen from *Staphylococcus epidermidis*. FEMS Immunol Med Microbiol 29:213–220

Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ (2009) Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. Proteomics 9:1696–1719

Liduma I, Tracevska T, Bers U, Zilevica A (2012) Phenotypic and genetic analysis of biofilm formation by *Staphylococcus epidermidis*. Medicina (Kaunas) 48:305–309

Mack D, Becker P, Chatterjee I, Dobinsky S, Knobloch JK, Peters G, Rohde H, Herrmann M (2004) Mechanisms of biofilm formation in *Staphylococcus epidermidis* and *Staphylococcus aureus*: functional molecules, regulatory circuits, and adaptive responses. Int J Med Microbiol 294:203–212

Martin-Lopez JV, Diez-Gil O, Morales M, Batista N, Villar J, Claverie-Martin F, Mendez-Alvarez S (2004) Simultaneous PCR detection of ica cluster and methicillin and mupirocin resistance genes in catheter-isolated *Staphylococcus*. Int Microbiol 7:63–66

Molloy MP, Herbert BR, Slade MB, Rabilloud T, Nouwens AS, Williams KL, Gooley AA (2000) Proteomic analysis of the *Escherichia coli* outer membrane. Eur J Biochem 267: 2871–2881

Oosthuizen MC, Steyn B, Theron J, Cosette P, Lindsay D, Von Holy A, Brozel VS (2002) Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. Appl Environ Microbiol 68:2770–2780

Planchon S, Desvaux M, Chafsey I, Chambon C, Leroy S, Hebraud M, Talon R (2009) Comparative subproteome analyses of planktonic and sessile *Staphylococcus xylosus* C2a: new insight in cell physiology of a coagulase-negative *Staphylococcus* in biofilm. J Proteome Res 8:1797–1809

Resch A, Rosenstein R, Nerz C, Gotz F (2005) Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. Appl Environ Microbiol 71:2663–2676

Resch A, Leicht S, Saric M, Pasztor L, Jakob A, Gotz F, Nordheim A (2006) Comparative proteome analysis of *Staphylococcus aureus* biofilm and planktonic cells and correlation with transcriptome profiling. Proteomics 6:1867–1877

Rohde H, Burdelski C, Bartscht K, Hussain M, Buck F, Horstkotte MA, Knobloch JK, Heilmann C, Herrmann M, Mack D (2005) Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Mol Microbiol 55:1883–1895

Rupp ME, Ulphani JS, Fey PD, Mack D (1999a) Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. Infect Immun 67:2656–2659

Rupp ME, Ulphani JS, Fey PD, Bartscht K, Mack D (1999b) Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. Infect Immun 67:2627–2632

Sanchez CJ Jr, Mende K, Beckius ML, Akers KS, Romano DR, Wenke JC, Murray CK (2013) Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infect Dis 13:47

Schumacher-Perdreau F, Heilmann C, Peters G, Gotz F, Pulverer G (1994) Comparative analysis of a biofilm-forming *Staphylococcus epidermidis* strain and its adhesion-positive, accumulation-negative mutant M7. FEMS Microbiol Lett 117:71–78

Schwank S, Rajacic Z, Zimmerli W, Blaser J (1998) Impact of bacterial biofilm formation on in vitro and in vivo activities of antibiotics. Antimicrob Agents Chemother 42:895–898

Seyer D, Cosette P, Siroy A, Dé E, Lenz C, Vaudry H, Coquet L, Jouenne T (2005) Proteomic comparison of outer membrane protein patterns of sessile and planktonic *Pseudomonas aeruginosa* cells. Biofilms 2:27–36

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850–858

Shin JH, Lee HW, Kim SM, Kim J (2009) Proteomic analysis of *Acinetobacter baumannii* in biofilm and planktonic growth mode. J Microbiol 47:728–735

Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ, McKenna T, Nold MJ, Richardson K, Young P, Geromanos S (2005) Quantitative proteomic analysis by accurate mass retention time pairs. Anal Chem 77:2187–2200

Silva JC, Gorenstein MV, Li GZ, Vissers JP, Geromanos SJ (2006) Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. Mol Cell Proteomics 5:144–156

Siroy A, Cosette P, Seyer D, Lemaître-Guillier C, Vallenet D, Van Dorsselaer A, Boyer-Mariotte S, Jouenne T, Dé E (2006) Global comparison of the membrane subproteomes between a multidrug-resistant *Acinetobacter baumannii* strain and a reference strain. J Proteome Res 5:3385–3398

Soares NC, Cabral MP, Parreira JR, Gayoso C, Barba MJ, Bou G (2009) 2-DE analysis indicates that *Acinetobacter baumannii* displays a robust and versatile metabolism. Proteome Sci 7:37

Stevens NT, Greene CM, O'Gara JP, Humphreys H (2009) Biofilm characteristics of *Staphylococcus epidermidis* isolates associated with device-related meningitis. J Med Microbiol 58:855–862

Steyn B, Oosthuizen MC, MacDonald R, Theron J, Brozel VS (2001) The use of glass wool as an attachment surface for studying phenotypic changes in *Pseudomonas aeruginosa* biofilms by two-dimensional gel electrophoresis. Proteomics 1:871–879

Sun D, Accavitti MA, Bryers JD (2005) Inhibition of biofilm formation by monoclonal antibodies against *Staphylococcus epidermidis* RP62A accumulation-associated protein. Clin Diagn Lab Immunol 12:93–100

Szczuka E, Kaznowski A (2014) Antimicrobial activity of tigecycline alone or in combination with rifampin against *Staphylococcus epidermidis* in biofilm. Folia Microbiol 59:283–288

Szczuka E, Urbanska K, Pietryka M, Kaznowski A (2013) Biofilm density and detection of biofilm-producing genes in methicillin-resistant *Staphylococcus aureus* strains. Folia Microbiol (Praha) 58:47–52

Tan S, Tan HT, Chung MC (2008) Membrane proteins and membrane proteomics. Proteomics 8:3924–3932

van Alen T, Claus H, Zahedi RP, Groh J, Blazyca H, Lappann M, Sickmann A, Vogel U (2010) Comparative proteomic analysis of biofilm and planktonic cells of *Neisseria meningitidis*. Proteomics 10:4512–4521

Visutthi M, Srimanote P, Voravuthikunchai SP (2011) Responses in the expression of extracellular proteins in methicillin-resistant *Staphylococcus aureus* treated with rhodomyrtone. J Microbiol 49:956–964

Wisniewski JR, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation method for proteome analysis. Nat Methods 6:359–362

World Health Organization (2004) Laboratory biosafety manual, 3rd edn. WHO, Geneva

Yan L, Zhang L, Ma H, Chiu D, Bryers JD (2014) A single B-repeat of *Staphylococcus epidermidis* accumulation-associated protein induces protective immune responses in an experimental biomaterial-associated infection mouse model. Clin Vaccine Immunol 21:1206–1214

Yang XM, Li N, Chen JM, Ou YZ, Jin H, Lu HJ, Zhu YL, Qin ZQ, Qu D, Yang PY (2006) Comparative proteomic analysis between the invasive and commensal strains of *Staphylococcus epidermidis*. FEMS Microbiol Lett 261:32–40

Zhang YQ, Ren SX, Li HL, Wang YX, Fu G, Yang J, Qin ZQ, Miao YG, Wang WY, Chen RS, Shen Y, Chen Z, Yuan ZH, Zhao GP, Qu D, Danchin A, Wen YM (2003) Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). Mol Microbiol 49:1577–1593