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An immunoproteomic approach for characterization of dormancy within *Staphylococcus epidermidis* biofilms

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ABSTRACT

Virulence of *Staphylococcus epidermidis* is mainly attributed to surface colonization and biofilm formation in indwelling medical devices. Physiological heterogeneity of biofilms may influence host immune response and sensitivity to antibiotics. Dormant cells, among others, contribute to biofilm heterogeneity. The aim of this study was to identify immunogenic proteins of *S. epidermidis* biofilms associated with dormancy mechanism, by using two-dimensional electrophoresis (2-DE) immunoblotting and mass spectrometry (MS). A total of 19 bacterial proteins, recognized by human serum samples, were identified. These proteins were mainly involved in small molecule metabolic biological processes. Catalytic activity and ion binding were the most representative molecular functions. CodY and GpmA proteins were more reactive to sera when biofilm dormancy was induced, while FtnA and ClpP were more reactive when dormancy was prevented. This is the first work that identifies differences in immunoreactive proteins within bacterial biofilms with induced or prevented dormancy. Considering the importance of dormancy within biofilms, further evaluation of these proteins can provide insights into the mechanisms related to dormancy and help to improve current understanding on how dormancy affects the host immune response.

1. Introduction

Staphylococcus epidermidis is an important opportunistic bacterium that does not produce highly aggressive virulence determinants (Otto, 2009). Its main virulence factor is the ability to form biofilms in indwelling medical devices (Otto, 2014). Biofilms are a community of surface-attached bacteria surrounded by an extracellular polymeric matrix composed of substances such as DNA, polysaccharides and proteins (Costerton et al., 1999). The clinical implications of bacterial growth in a biofilm mode are higher tolerance to antibiotics (Cerca et al., 2005) and tolerance to the innate immune response (Gray et al., 1984; Yao et al., 2005; Cerca et al., 2014; Cerca et al., 2006). *S. epidermidis* biofilm evasion of the host immune response may be caused by the production of several molecules that provide protection to host defenses, such as proteins, exopolysaccharides and peptides with antimicrobial activity (Otto, 2012). Biofilm protection against components of the innate immune mechanisms (Vuong et al., 2004; Jesaitis et al., 2003; Leid et al., 2002), such as phagocytosis (Johnson et al., 1986) and activity of antimicrobial peptides (Kristian et al., 2008; Vuong et al., 2004), is mainly mediated by the extracellular polymeric matrix (Cerca et al., 2006). In *S. epidermidis* biofilms, polysaccharide intercellular adhesin (PIA), also named poly-N-acetylglucosamine (PNAG) is considered a major virulence factor in biomaterial associated infections (Rupp et al., 1999).

Nowadays, proteomic approaches are contributing to elucidate the immunological response to microorganisms (Fulton and Twine, 2013). Immunoproteomics allows the identification of immunogenic and immunoreactive proteins that may participate in host–pathogen interactions and in host immune response (Dennehy and McClean, 2012; Costa et al., 2013; Wang et al., 2013). Furthermore, immunoproteome analysis improves the understanding of pathogenesis and unravel novel therapeutics targets based on the repertoire of immunogens (Brady et al., 2006).

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Thus, plasma is one of the most relevant environmental factors in indwelling medical devices-related infections (Schuster et al., 2014). A few aspects of immune reaction to *S. epidermidis* infections were already elucidated (Sellman et al., 2005; Franca et al., 2014; Cheung et al., 2010; Hanke and Kielian, 2012; Scherr et al., 2014; Pourmand et al., 2006). By using serum from rabbits immunized with live *S. epidermidis*, or serum proteins eluted from the surface of bacteria grown in rabbit serum reactive against bacterial cell-surface extracts, immunogenic and serum binding proteins were identified by Western blotting (Sellman et al., 2005). Sellman and colleagues found 5 antigenic components candidates for the development of *S. epidermidis* vaccine, namely, acetyl-coenzyme A acetyltransferase (YqiL), Na^+/H^+ antiporter (SE1873), lipoate ligase (SE0360), cysteine synthase (CysK), and alanine dehydrogenase (Ald). Also, Pourmand et al. identified autolysin AtlE, lipase (GehD) and surface protein ScaB antigenic components with therapeutic potential since they had opsonic activity *in vitro* (Pourmand et al., 2006).

Mature biofilms encompass cells with different metabolic activity (Rani et al., 2007), including dormant cells (Cerca et al., 2011). Dormancy is defined by a physiological state where bacteria persist without division for extended periods (Kaprelyants et al., 1993; Lewis, 2007). Moreover, dormant bacteria are associated with higher tolerance to antibiotics (Williamson et al., 2012; Kim et al., 2009; Shapiro et al., 2011; Cerca et al., 2014) and may determine the inflammatory profile of a biofilm (Cerca et al., 2011, 2014). Previously, we developed an *in vitro* model to modulate dormancy within *S. epidermidis* biofilms (Cerca et al., 2011). We were able to show that *S. epidermidis* biofilms with higher proportions of dormant bacteria induced a lower activation of murine macrophages, since it reduced *in vitro* pro-inflammatory cytokine production and lead to decreased expression of surface activation markers *in vivo* (Cerca et al., 2011). More recently, we performed a global transcriptome analysis where we found that translation was downregulated in dormant biofilms and, oxidation–reduction processes were associated with dormancy (Carvalhais et al., 2014). We also performed a quantitative proteomic analysis, where the ribosome synthesis pathway was associated with prevented dormancy, and ion binding and catalytic activity were found overexpressed in dormancy (Carvalhais et al., 2015).

To determine the immunoproteomic pattern of *S. epidermidis* biofilms with prevented and induced dormancy, we resolved whole cell lysate by 2-dimensional gel electrophoresis (2-DE) and performed immunoblotting with human sera. We then identified the immunoreactive protein spots by MALDI-TOF/TOF. With this work we intend to define the reactive protein repertoire of *S. epidermidis* biofilms with different proportions of dormant bacteria to human serum and contribute to decipher the host immune differences to dormancy.

2. Materials and methods

2.1. Growth conditions

Growth culture condition was performed as previously described (Cerca et al., 2011). *S. epidermidis* strain 9142 (isolated from blood culture (Mack et al., 1992)) was used to establish biofilms with higher and lower ratios of dormant cells. Briefly, one colony of *S. epidermidis* was inoculated in Tryptic Soy Broth (TSB) (LiofilChem, Roseto Degli Abruzzi, Italy) and incubated at 37 °C in an orbital shaker at 120 rpm for 18 h. The overnight culture was adjusted to an optical density at 640 nm of 0.250 (±0.05) and 10 μL of the suspension was transferred into a 24-well plate (Orange Scientific, Braine-l'Alleud, Belgium) containing 1 mL of TSB supplemented with 0.4% glucose (v/v) (TSB 0.4% G) (Fisher Scientific, Waltham, MA, USA) or TSB 0.4% G enriched with 20 mM magnesium chloride ($MgCl_2$) (Merck, Darmstadt, Germany). The culture plates were then incubated at 37 °C in an orbital shaker at 120 rpm for 24 h. After this period, the culture medium was removed and replaced by fresh TSB supplemented with 1% glucose (v/v) (1% G) or TSB 1% G containing 20 mM $MgCl_2$ (1% G + Mg^{2+}). Biofilms were then allowed to grow in the same conditions for an additional 24 h. Next, biofilm culture medium was removed and biofilms were washed twice with phosphate buffered saline (PBS). Then, bacteria within the biofilms were resuspended in 1 mL of PBS. As previously described, biofilm dormancy was determined using the spread plate method in Trypticase Soy Agar (LiofilChem) through calculation of the number of CFU/mL in each biofilm growth condition (Cerca et al., 2011). A reduction of about one log difference is typically expected in similarly grown biofilms without Mg^{2+} (Cerca et al., 2011).

2.2. Preparation of protein extracts

Total protein extraction was performed from multiple biofilm replicates, as previously described (Carvalhais et al., 2015). Briefly, biofilms were directly scrapped and resuspended with detergent extraction buffer, consisting of 25 mM Tris–HCl (pH = 7.2) (Pharmacia Biotech, Uppsala, Sweden), 10 mM CHAPS (Sigma–Aldrich, St. Louis, MO, USA), 0.5 M NaCl (VWR, Radnor, PA, USA), 5% glycerol (Sigma–Aldrich) and 1 mM PMSF (Sigma–Aldrich). Then, mechanical lysis was performed in a bead beating using glass beads of 0.1 mm (Sigma–Aldrich) in a FastPrep® cell disruptor (BIO 101, ThermoElectron Corporation) (3 cycles of 30 s and a speed of 6.5 m/s). After lysis, cell debris was removed by centrifugation (15,000 × *g* for 15 min at 4 °C). Proteins were precipitated with 20% of trichloroacetic acid (TCA)-cold acetone. The lysates were mixed with 20% TCA (Sigma–Aldrich) and incubated for 60 min at −20 °C. Proteins were collected by centrifugation and washed three times with cold acetone. After drying, proteins were directly resuspended in 1% CHAPS, 8 M urea (Amersham Biosciences, Piscataway, NJ, USA), 2 M thiourea (Riedel-de Haën, Sigma–Aldrich) and 12 mM DTT (USB Corporation, Cleveland, OH, USA). Total protein was quantified using the RC-DC assay (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions.

2.3. Two-dimensional electrophoresis (2-DE)

A total of 80 μg of protein was resuspended in rehydration sample buffer (8 M urea, 2 M thiourea, 1% CHAPS, 12 mM DTT, 0.5% IPG buffer). Then, immobilized pH gradient (IPG) 3–10 non-linear strips, 7 cm, (Immobiline™ pH Gradient, GE Healthcare) were in-gel rehydrated overnight for the first dimension isoelectric focusing (IEF), performed on a horizontal Ettan™ IPGPhor (Amersham Biosciences, USA). Isoelectric separation was performed using the following focusing program: 12 h at 50 mW (rehydration), 1 h at 150 V (gradient), 1 h at 500 V (gradient), 1 h at 1000 V (gradient) and 90 min at 5000 V ("step-and-hold"). After IEF, IPG strips were equilibrated with equilibration buffer (2% (w/v) SDS, 6 M urea, 30% glycerol, 0.05 M Tris–HCl pH 8.8 and 20 mg/ml DTT) for 30 min at room temperature. Strips were then placed on the top of a 12% SDS-PAGE gel for the second dimension separation and ran at a constant voltage. Gels were stained with colloidal Coomassie G-250 or gels were transferred onto a nitrocellulose membrane. Proteins were blotted on a nitrocellulose membrane (Whatman®, Protan) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3 and 20% methanol) during 2 h at 200 mA. Stained gels were analyzed by using Melanie analysis software v.7.0 (GeneBio, Switzerland). Protein separations by 2-DE was carried out three independent times. The signal intensities of proteins spots were compared among both conditions and scored by fold-change.