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Research article

Identification of pathogenic factors potentially involved in *Staphylococcus aureus* keratitis using proteomics

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ABSTRACT

Staphylococcus is a leading cause of microbial keratitis, characterized by destruction of the cornea by bacterial exoproteins and host-associated factors. The aim of this study was to compare extracellular and cell-associated proteins produced by two different isolates of *S. aureus*, a virulent clinical isolate (Staph 38) and a laboratory strain (*Staphylococcus aureus* 8325-4) of weaker virulence in the mouse keratitis model. Proteins were analyzed using 2D polyacrylamide gel electrophoresis and identified by subsequent mass spectrometry. Activity of staphylococcal adhesins was assessed by allowing strains to bind to various proteins adsorbed onto polymethylmethacrylate squares. Thirteen proteins in the extracellular fraction and eight proteins in the cell-associated fractions after bacterial growth were produced in increased amounts in the clinical isolate Staph 38. Four of these proteins were *S. aureus* virulence factor adhesins, fibronectin binding protein A, staphopain, glyceraldehyde-3-phosphate dehydrogenase 2 and extracellular adherence protein. The clinical isolate Staph 38 adhered to a greater extent to all mammalian proteins tested, indicating the potential of the adhesins to be active on its surface. Other proteins with increased expression in Staph 38 included potential moonlighting proteins and proteins involved in transcription or translation. This is the first demonstration of the proteome of *S. aureus* isolates from keratitis. These results indicate that the virulent clinical isolate produces more potentially important virulence factors compared to the less virulent laboratory strain and these may be associated with the ability of a *S. aureus* strain to cause more severe keratitis.

1. Introduction

Staphylococcus has remained one of the leading causes of microbial keratitis worldwide for over twenty years (Asbell and Stenson, 1982; Hernandez-Camarena et al., 2015; Lin et al., 2015; Ng et al., 2015; Ni et al., 2015; Sand et al., 2015; Simcock et al., 1996), with predisposing factors including contact lens wear and immunocompromised individuals such as those with HIV, diabetes or aged populations. Staphylococcal keratitis is characterized by the destruction of the cornea by bacterial exoproteins and host-associated factors (Hume et al., 2005; O'Callaghan et al., 1997).

Treatment of *Staphylococcus aureus* infections is extremely labor-intensive and usually involves the application of antibiotic drops every half-hour for 48 h or longer. Additionally, *S. aureus* is particularly difficult to treat due to its ability to acquire resistance to many antibiotics. Methicillin-resistant strains of *S. aureus* (MRSA) are common for keratitis isolates (Schubert et al., 2008), with studies showing rates of MRSA from 25% to 45% of cases (Hernandez-Camarena et al., 2015; Sand et al., 2015). Alarmingly, increasing resistance to vancomycin, which is currently the drug of choice for MRSA in many infections, has been reported (Hernandez-Camarena et al., 2015; Kantzanou et al., 1999) as has resistance to fluoroquinolones which are often used as a first line therapy for microbial keratitis (Marangon et al., 2004; Ni et al., 2015). Despite the ability to dose relatively high concentrations of antibiotics to the cornea by topical application, antibiotic resistance still affects clinical outcome as antibiotic resistant infections increase infection recovery time (Wilhelmus et al., 2003). Consequently, an understanding of host-pathogen interactions is crucial

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to the development of effective strategies for the prevention and treatment of *S. aureus*.

A topical mouse model of *S. aureus* keratitis has been developed and the cytokine response to *S. aureus* has been characterized (Hume et al., 2005), and a protective role for the cytokines IL-6 and IL-4 has been shown (Cole et al., 2007; Hume et al., 2006). In this topical keratitis model, a clinical microbial keratitis isolate, Staph 38, was more virulent than the standard laboratory isolate *S. aureus* 8325-4 (Hume et al., 2005). Corneas infected with the clinical isolate showed significant loss of epithelium and an increase in the number of infiltrating white blood cells compared to corneas infected with the laboratory isolate *S. aureus* 8325-4 (Hume et al., 2005). This is important as the laboratory strain has been extensively used in infection models as well as in the rabbit model of keratitis (O'Callaghan et al., 1997). In addition to being more virulent, the ability of Staph 38 to invade corneal epithelial cells was approximately 250-fold greater than that of the laboratory isolate *S. aureus* 8325-4 (Hume et al., 2005).

Although alpha toxin produced by *S. aureus* has been shown to be a major virulence factor in the rabbit intra-stromal injection model of keratitis (Callegan et al., 1994; Hume et al., 2000; O'Callaghan et al., 1997; Weeks et al., 2012), the clinical isolate Staph 38 was found to produce less alpha-toxin than the laboratory strain *S. aureus* 8325-4 (Hume et al., 2005). In other models of non-ocular infections *S. aureus* produces a number of important extracellular and cell-associated virulence factors. Cell-associated factors that have been linked with increased virulence in various infections include members of the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family (Foster et al., 2014) such as fibronectin binding proteins A (FnBPA) and B (FnBPB) (Peacock et al., 2000), and the collagen-adhesin, which has been shown to be involved in keratitis (Rhem et al., 2000). Other proteins that have been shown to be involved in non-ocular infections include proteins that enhance invasion of mammalian cells such as iron-regulated surface determinant B and extracellular adherence protein (Haggar et al., 2003; Zapotoczna et al., 2013), and cell wall-associated glyceraldehyde-3-phosphate dehydrogenase which binds transferrin and is involved in biofilm formation (Foulston et al., 2014; Modun and Williams, 1999). Extracellular virulence factors involved in non-ocular infections include a variety of other cytotoxins and proteases (Otto, 2014; Shaw et al., 2004). *S. aureus* also produces protein A, a cell surface protein involved in immune evasion (Foster et al., 2014).

It is likely that differing expression of such virulence factors may also influence the outcome of corneal infection. In this study we examined differences in the extracellular and cell-associated proteomes of two strains of *S. aureus*, which differ in virulence in an experimental model of corneal infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Laboratory type strain *S. aureus* 8325-4 which is a derivative of the strain NCTC 8325 cured of prophages with an 11-bp deletion in rsbU (Horsburgh et al., 2002) (kindly provided by Professor R O'Callaghan) and a clinical isolate from a human corneal ulcer *S. aureus* 38 were used in this study (Hume et al., 2005). Strains were stored at −80 °C in 30% glycerol v/v in Tryptone Soy Broth (TSB; v/v, Oxide, Basingstoke, UK) prior to use.

2.2. Isolation of extracellular and cell-associated proteins

The two strains (Staph 38 and *S. aureus* 8325-4) were grown in TSB at 37 °C to mid-exponential phase. The bacterial cells were pelleted (6000*g*, 4 °C, 10 min) for extraction of cell-associated proteins, and the supernatant was dialyzed to remove the TSB components. The bacterial pellet was freeze-dried prior to extraction of the cell-associated proteins (Cordwell et al., 2002). Briefly, 10 mg of freeze-dried cells were resuspended in MiPrep M sample buffer (Minomic, Sydney, NSW, Australia) and solubilized by probe sonication (5 × 20s bursts) on ice with 2 min intervals. Cellular debris was removed by centrifuging (6000*g*, 3 min, 24 °C). *Serratia marcescens* endonuclease (150 U, Sigma-Aldrich, St Louis, MO, USA) and bacterial protease cocktail inhibitor (1 unit 2 μL, Sigma-Aldrich) were added to the resulting cell lysate, or to the dialyzed supernatants. To obtain extracellular proteins, supernatants from exponential phase cells were extracted as previously described by Nouwens et al. (2000). Dialyzed supernatants were freeze-dried and the extracellular proteins (10–30 mg) rehydrated in sample buffer MiPrep M (Minomic) and sonicated twice for 5 s on ice to solubilize the proteins.

2.3. 2D gel electrophoresis

All chemicals, unless otherwise noted, were purchased from Sigma-Aldrich. For iso-electric focusing, samples containing 100 μg of protein were diluted to 300 μL in MiPrepM sample buffer (Minomic, Sydney, Australia) and loaded into 11 cm 4–7 pH and 6–11 pH IPG precast gel strips (Bio-Rad, CA, USA) and focused for 70 kVh. After focusing, strips were equilibrated in reducing solution (Urea 6 M, 2% SDS, 20% glycerol (v/v), 65 mM DTT) for 15 min then equilibrated in alkylating solution (Urea 6 M, 2% SDS, 20% glycerol (v/v), 2.5% acrylamide, pH 8.8) for 15 min. For the second dimension, IPG strips were loaded onto 8–16% (11 cm × 20 cm) Tris-HCL gels (Bio-Rad) in Tris-HCL buffer (0.37 M Tris pH8.8) with 0.5% agarose with bromophenol blue. Electrophoresis was carried out at 4 °C, 3 mA gel^{-1} for 1 h. The gels were fixed with 10% ethanol and 10% acetic acid for 1 min in a microwave (1100 Watts) before staining in Sypro Ruby (Molecular Probes, Invitrogen, CA, USA) for 1 min in a microwave on full power. The gels were then destained in the fixing solution for 1 min in a microwave on full power. Gels were stained in Coomassie-blue G250 (0.1% w/v in 17% ammonium sulfate, 34% methanol and 3% orthophosphoric acid) overnight. Gels were then destained in 6% acetic acid and immersed in a solution of 1% acetic acid for image analysis and protein identification.

2.4. Image analysis

All gels were repeated a minimum of five times to confirm any differences between strains. When proteins were absent, additional gels were overloaded (5× concentration) and run to ensure that undetected proteins were not a result of detection limits. Progenesis (Non Linear Dynamics, Newcastle, UK) was used to identify protein spots that were more abundant or only produced in the clinical isolate Staph 38 compared to the laboratory strain *S. aureus* 8325-4. An average normalized volume for each gel spot was produced from the five imaged gels. Only those gel spots appearing on all five gels were analyzed. Increases in spot intensity of greater than 3-fold were selected for protein identification using peptide mass fingerprinting. Data was analyzed using Analysis of Variance (SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.).

2.5. Protein matching using peptide mass mapping

Protein spots of increased abundance in the clinical isolate were excised and further analyzed by matrix assisted laser ionization/time of flight (MALDI/TOF). If identification by this method was unsuccessful the protein spot sample was further analyzed by liquid chromatography tandem mass spectrometry (LC/MS-MS).