Veterinary Microbiology 138 (2009) 345–352

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Staphylococcus pseudintermedius expresses surface proteins that closely resemble those from *Staphylococcus aureus*

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ARTICLE INFO

Article history:
Received 27 November 2008
Received in revised form 12 March 2009
Accepted 19 March 2009

Keywords:
Staphylococcus pseudintermedius
Surface protein
Adhesion

ABSTRACT

Staphylococcus pseudintermedius is a commensal of dogs that is implicated in the pathogenesis of canine pyoderma. This study aimed to determine if *S. pseudintermedius* expresses surface proteins resembling those from *Staphylococcus aureus* and to characterise them. *S. pseudintermedius* strain 326 was shown to adhere strongly to purified fibrinogen, fibronectin and cytokeratin 10. It adhered to the α-chain of fibrinogen which, along with binding to cytokeratin 10, is the hallmark of clumping factor B of *S. aureus*, a surface protein that is in part responsible for colonisation of the human nares. Ligand-affinity blotting with cell-wall extracts demonstrated that *S. pseudintermedius* 326 expressed a cell-wall anchored fibronectin binding protein which recognised the N-terminal 29 kDa fragment. The ability to bind fibronectin is an important attribute of pathogenic *S. aureus* and is associated with the ability of *S. aureus* to colonise skin of human atopic dermatitis patients. *S. pseudintermedius* genomic DNA was probed with labelled DNA amplified from the serine-aspartate repeat encoding region of *clfA* of *S. aureus*. This probe hybridised to a single *Spe*I fragment of *S. pseudintermedius* DNA. In the cell-wall extract of *S. pseudintermedius* 326, a 180 kDa protein was discovered which bound to fibrinogen by ligand-affinity blotting and reacted in a Western blot with antibodies raised against the serine-aspartate repeat region of ClfA and the B-repeats of SdrD of *S. aureus*. It is proposed that this is an Sdr protein with B-repeats that has an A domain that binds to fibrinogen. Whether it is the same protein that binds cytokeratin 10 is not clear.

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1. Introduction

Staphylococcus pseudintermedius (until recently called *S. intermedius*) is a commensal of healthy dogs (Bannoehr et al., 2007; Devriese et al., 2005). It can also infect the skin of dogs suffering from atopic dermatitis causing pyoderma. The ability of *S. aureus* to adhere to desquamated epithelial cells is an important correlate of colonisation of the nares of humans (Wertheim et al., 2008). Clumping factor B (ClfB) and iron-regulated surface determinant protein IsdA play important roles in adhesion to squamous cells and colonisation of the nares of rodents, and in the case of ClfB, humans (Clarke et al., 2006; Schaffer et al., 2006; Wertheim et al., 2008). ClfB binds to cytokeratin 10 (O'Brien et al., 2002; Walsh et al., 2004) which is expressed on the surface of squamous epithelial cells where it presumably provides a ligand for bacterial attachment. ClfB also binds to the α-chain of fibrinogen in contrast to other fibrinogen binding surface proteins which bind to the β-chain or the γ-chain (Davis et al., 2001; McDevitt et al., 1997; Walsh et al., 2008; Wann et al., 2000). *S. pseudintermedius* adheres to canine corneocytes so it is reasonable to predict similar mechanisms of adhesion as for *S. aureus* (McEwan, 2000).

S. pseudintermedius adheres more strongly to corneocytes from regions of inflamed skin from a dog with atopic

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doi:10.1016/j.vetmic.2009.03.030

dermatitis than to non-inflamed areas suggesting that ligands for bacterial surface proteins are expressed at higher levels (McEwan et al., 2006). In addition, fibronectin is present at elevated levels in the *stratum corneum* of atopic human skin whereas it was not detected in healthy skin (Cho et al., 2001). This could provide a receptor for fibronectin binding proteins of *S. aureus*.

Given the similarity of *S. pseudintermedius* and *S. aureus* and the fact that both organisms adhere to squamous cells from their respective hosts as well as infect inflamed skin in atopic dermatitis it seems reasonable to expect that *S. pseudintermedius* would display a repertoire of surface proteins similar to those of *S. aureus*. This study aimed to determine if *S. pseudintermedius* adheres to fibrinogen, fibronectin, elastin and cytokeratin 10 and to characterise the surface proteins responsible.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. pseudintermedius strains used in this study were isolated from cases of canine pyoderma and were a kind gift from Dr. Neil McEwan, University of Liverpool. *S. aureus* strain Newman is a human clinical isolate (Duthie and Lorenz, 1952), Newman *clfB* is a mutant deficient in clumping factor B (Ni Eidhin et al., 1998) and Newman *clfA clfB* lacks clumping factor A and ClfB (Fitzgerald et al., 2006). *S. aureus* SH1000 shows strong adherence to extracellular matrix proteins and is a derivative of strain 8325-4 with a repaired defect in *rsbU* (Horsburgh et al., 2002). *S. aureus* P1 adheres strongly to fibronectin (Fitzgerald et al., 2006; Roche et al., 2004; Sherertz et al., 1993).

Strains were grown in brain heart infusion (BHI, Oxoid) broth at 37 °C with aeration. Stationary phase cultures were grown for approximately 16 h. Exponential phase cultures were inoculated 1:100 from overnight starter cultures. Cells were washed in BHI and grown to an optical density of 0.6.

2.2. Ligand and Western immunoblot analysis

Exponential or stationary phase cultures were harvested, washed in phosphate-buffered saline (PBS) and resuspended to OD_{600} of 40 in lysis buffer (50 mM Tris/HCl, 20 mM $MgCl_2$, pH 7.5) supplemented with 30% (w/v) raffinose and complete protease inhibitors (40 μl/ml, Roche). Cell-wall proteins were solubilised by incubation with lysostaphin (200 μg/ml; AMBI, New York) for 10 min at 37 °C. Protoplasts were removed by centrifugation at 12,000 × *g* for 10 min and the supernatant containing solubilised cell-wall proteins was aspirated and boiled for 5 min in final sample buffer (0.125 M Tris/HCl, 4%, w/v, SDS, 20% glycerol, 10%, v/v, 2-mercaptoethanol, 0.002%, w/v, bromphenol blue). Proteins were separated on 7.5% (w/v) polyacrylamide gels and electrophoretically transferred onto PVDF membranes (Roche) and blocked in 10% (w/v) skimmed milk (Marvel).

Blots were probed with anti-ClfA SD-repeat antibodies (1:1000; a gift from O. Hartford, Trinity College, Dublin), anti-SdrD B-repeat antibodies (1:1000; a gift from L. O'Brien, Trinity College, Dublin) or fibrinogen (20 μg/ml, Calbiochem). Bound antibodies were detected using horseradish peroxidase-conjugated (HRP) protein A (1:500; Sigma) and bound fibrinogen was detected with HRP-conjugated anti-fibrinogen antibody (1:3000, Dako). Biotinylated fibronectin was used in ligand-affinity blots. Human fibronectin (0.5 mg/ml in PBS) was incubated with biotin (2 mg/ml) for 20 min at room temperature. The reaction was stopped by the addition of 10 mM NH_4Cl. Excess biotin was removed by dialysis against PBS overnight at 4 °C. Blots were probed with biotinylated fibronectin and POD-conjugated streptavidin (1:5000; Roche). Reactive bands were visualised using the LumiGLO reagent and peroxide detection system (Cell Signalling Technology).

Filters to be reprobed with another antibody were stripped using a solution of 2% (w/v) SDS, 100 mM β-mercaptoethanol and 50 mM Tris at 50 °C for 30 min, washed twice for 10 m in TS (10 mM Tris-HCl, 0.9% (w/v) NaCl, pH 7.4) buffer and then blocked in 10% Marvel for 2–18 h.

2.3. Bacterial adherence to fibrinogen and fibronectin

Microtitre plates (Sarstedt) were coated with doubling dilutions of human fibrinogen (Calbiochem), canine fibrinogen (Sigma) or human fibronectin (Calbiochem) in PBS. Plates were coated overnight at 4 °C and blocked for 2 h at 37 °C with 5% (w/v) bovine serum albumin (BSA). Washed exponential or stationary phase cells were adjusted to an OD_{600} of 1.0 in PBS, and 100 μl was added to each well and incubated for 2 h at 37 °C. After washing with PBS, adherent cells were fixed with formaldehyde (25%, v/v), stained with crystal violet and the A_{570} measured. Adherence assays with recombinant cytokeratin 10, recombinant fibrinogen α-chain (both gifts from H. Miajlovic, Trinity College, Dublin) and purified fibronectin N29 fragment were performed with Nunc microtitre plates using sodium carbonate buffer (pH 9.6) instead of PBS.

2.4. Bacterial adhesion to immobilised elastin peptides

Bacterial adhesion to immobilised elastin peptides was performed as previously described (Roche et al., 2004). Briefly, microtitre plate wells (Povair) were coated with doubling dilutions aortic elastin peptides (Elastin Products Company) in PBS and air dried under UV light (366 nM) at room temperature for 18 h. Wells were blocked for 2 h at 37 °C with 5% BSA. Bacteria were washed with PBS and resuspended to an OD = 1.0 (1×10^8 colony forming units ml^{-1}). Bacterial cell adherence was measured using a fluorescent nucleic acid stain SYTO-13 (Molecular Probes). Bacterial cells were incubated with SYTO-13 (5 μM) at room temperature for 15 min in the dark. Elastin-coated wells were washed three times with PBS and 100 μl of stained cells was added to the plate and incubated with shaking in the dark for 1 h. Wells were washed three times with PBS and adherent bacteria were measured using an LS-50B spectrophotometer (PerkinElmer) with excitation at 488 nm and emission at 509 nm.

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